(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 June 2002 (13.06.2002)

PCT

(10) International Publication Number
WO 02/46768 A2

(51) International Patent Classification[7]: G01N 33/68

(21) International Application Number: PCT/GB01/05476

(22) International Filing Date:
10 December 2001 (10.12.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0030051.7 8 December 2000 (08.12.2000) GB

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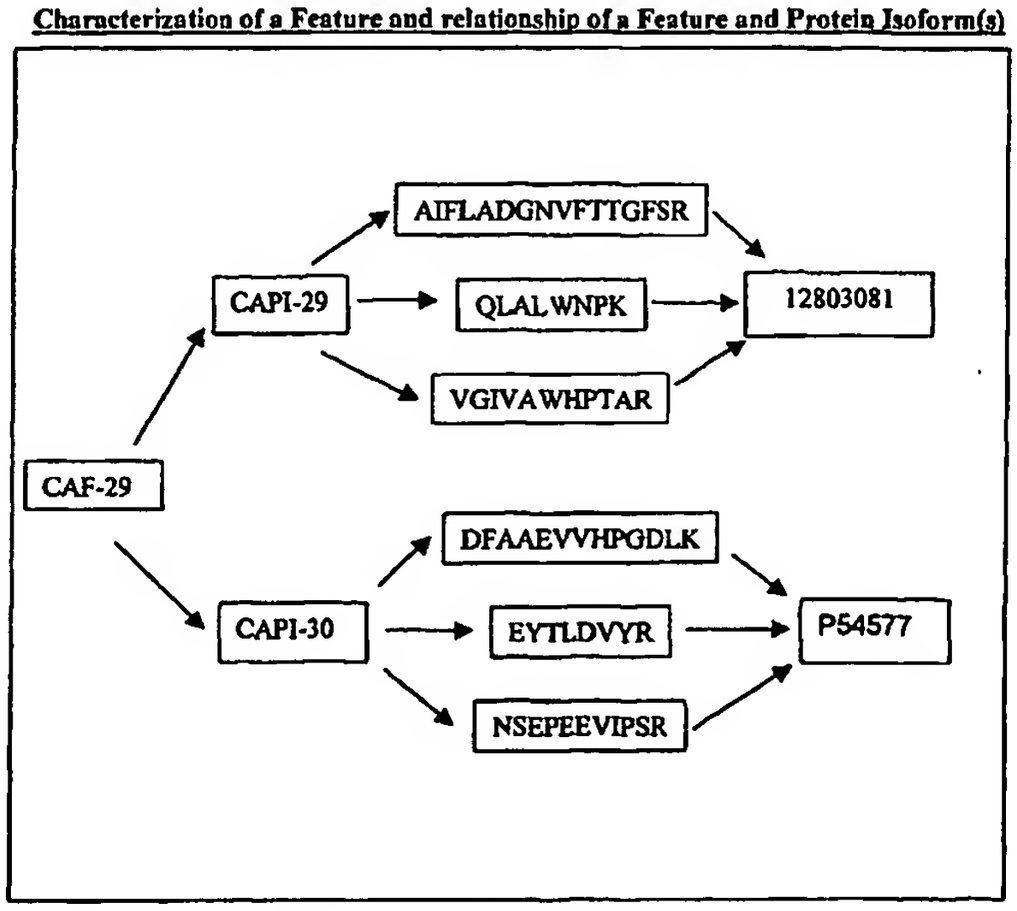
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Declaration under Rule 4.17:**
— *of inventorship (Rule 4.17(iv)) for US only*

*[Continued on next page]*

(54) Title: PROTEINS, GENES AND THEIR USE FOR DIAGNOSIS AND TREATMENT OF CHRONIC ASTHMA




(57) Abstract: The present invention provides methods and compositions for screening, diagnosis and prognosis of chronic asthma, for monitoring the effectiveness of chronic asthma treatment, and for drug development. Chronic Asthma-Associated Features (CAFs), detectable by two-dimensional electrophoresis of tissue are described. The invention further provides Chronic Asthma-Associated Protein Isoforms (CAPIs) detectable in tissue preparations comprising isolated CAPIs, antibodies immunospecific for CAPIs, and kits comprising the aforesaid.

WO 02/46768 A2

**Published:**

— *without international search report and to be republished upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

# PROTEINS, GENES AND THEIR USE FOR DIAGNOSIS AND TREATMENT OF CHRONIC ASTHMA

## 1. INTRODUCTION

The present invention relates to the identification of proteins and protein isoforms that are associated with chronic asthma and its onset and development, and of genes encoding the same, and to their use for *e.g.*, clinical screening, diagnosis, prognosis, therapy and prophylaxis, as well as for drug screening and drug development.

## 2. BACKGROUND OF THE INVENTION

Asthma is an increasing problem worldwide; estimates suggest that 5.1 million people in the UK, (1 in 13 adults and 1 in 8 children), are currently being treated for asthma. In addition there are approximately 74,000 emergency hospital admissions as a result of asthma each year.

Current therapies used for the treatment and prophylaxis of asthma includes long-acting bronchodilators for prevention of mild asthma (e.g. salmeterol, formoterol), short-acting bronchodilators for acute asthma attacks (e.g. Salbutamol, isoproterenol, terbutaline) and corticosteroids for more serious asthmatic conditions (e.g. beclomethasone, fluticasone, budesonide). Corticosteroids are able to act on the inflammatory response seen in asthma, however, the possible side effects including immunosuppression make them a less than ideal means of treatment.

The inflammation associated with asthma can lead to remodelling of the extracellular matrix including subepithelial fibrosis, mucous metaplasia, myofibroblast hyperplasia and myocyte hyperplasia and hypertrophy. It is this remodelling that is a major component of chronic asthma.

Although glucocorticoids may be partially effective in preventing long-term inflammation there are no proven therapies that prevent airway remodelling. Primary human airway myofibroblasts are critical cells in airway remodelling. Increases in cell number, increased matrix deposition and increased synthetic properties of these cells all contribute to the structural changes characteristic of airway remodelling. Proliferative signals provided by PDGF and thrombin can be inhibited by glucocorticoids and phosphodiesterase inhibitors. The present invention discloses downstream targets of glucocorticoids and/or theophylline in an attempt to identify novel targets for modulation of airway remodelling.

## 3. SUMMARY OF THE INVENTION

The present invention provides methods and compositions for clinical screening, diagnosis, prognosis, therapy and prophylaxis of chronic asthma, for monitoring the effectiveness of chronic asthma treatment, for selecting participants in clinical trials, for identifying patients most likely to respond to a particular therapeutic treatment and for screening and development of drugs for treatment of chronic asthma.

A first aspect of the invention provides methods for diagnosis of chronic asthma that comprise analyzing a bronchial biopsy by two-dimensional electrophoresis to detect the presence or level of at least one Chronic Asthma-Associated Feature (CAF), *e.g.*, one or more of the CAFs disclosed herein, or any combination thereof. These methods are also suitable for clinical screening, prognosis, monitoring the results of therapy, for identifying patients most likely to respond to a particular therapeutic treatment, drug screening and development, and identification of new targets for drug treatment.

A second aspect of the invention provides methods for diagnosis of chronic asthma that comprise detecting in a bronchial biopsy the presence or level of at least one Chronic Asthma-Associated Protein Isoform (CAPI), *e.g.*, one or more of the CAPIs disclosed herein or any combination thereof. These methods are also suitable for clinical screening, prognosis, monitoring the results of therapy, identifying patients

most likely to respond to a particular therapeutic treatment drug screening and development, and identification of new targets for drug treatment.

A third aspect of the invention provides antibodies, *e.g.*, monoclonal and polyclonal capable of immunospecific binding to a CAPI, *e.g.*, a CAPI disclosed herein.

A fourth aspect of the invention provides preparations comprising an isolated CAPI, *i.e.*, a CAPI free from proteins or protein isoforms having a significantly different isoelectric point or a significantly different apparent molecular weight from the CAPI.

A fifth aspect of the invention provides methods of treating chronic asthma, comprising administering to a subject a therapeutically effective amount of an agent that modulates (*e.g.*, upregulates or downregulates) the expression or activity (*e.g.* enzymatic or binding activity), or both, of a CAPI in subjects having chronic asthma, in order to prevent or delay the onset or development of chronic asthma, to prevent or delay the progression of chronic asthma, or to ameliorate the symptoms of chronic asthma.

A sixth aspect of the invention provides methods of screening for agents that modulate (*e.g.*, upregulate or downregulate) a characteristic of, *e.g.*, the expression or the enzymatic or binding activity, of a CAF, CAPI, a CAPI fragment, a CAPI-related polypeptide, or a CAPI fusion protein.

## 4. BRIEF DESCRIPTION OF THE FIGURE

Figure 1 is a flow chart depicting the characterization of a Chronic Asthma-Associated Feature (CAF) and relationship of a CAF and Chronic Asthma-Associated Protein Isoform (CAPI). A CAF may be further characterized as or by a CAPI having a particular peptide sequence associated with its pI and MW. As depicted herein, a CAF may comprise one or more CAPI(s), which have indistinguishable pIs and MWs using the Preferred Technology, but which have distinct peptide sequences. The peptide sequence(s) of the CAPI can be utilized to search database(s) for previously identified proteins comprising such peptide sequence(s), for which previously

identified protein it can be ascertained whether a commercially available antibody exists which may recognize the previously identified protein and/or a member of its protein family.

Figure 2 is an image obtained from 2-dimensional electrophoresis of a primary culture of human airway myofibroblasts, which has been annotated to identify fifteen landmark CAFs, designated MF 1 to MF 15.

## 5. DETAILED DESCRIPTION OF THE INVENTION

The invention described in detail below provides methods and compositions for clinical screening, diagnosis and prognosis of chronic asthma in a mammalian subject, for monitoring the results of chronic asthma therapy, for identifying patients most likely to respond to a particular therapeutic treatment and for drug screening and drug development. The invention also encompasses the administration of therapeutic compositions to a mammalian subject to treat or prevent chronic asthma. The mammalian subject may be a non-human mammal, but is preferably human, more preferably a human adult, *i.e.* a human subject at least 21 (more preferably at least 35, at least 50, at least 60, at least 70, or at least 80) years old. For clarity of disclosure, and not by way of limitation, the invention will be described with respect to the analysis of bronchial biopsies. However, as one skilled in the art will appreciate, based on the present description the assays and techniques described below can be applied to other types of samples, including serum, a tissue sample from a subject at risk of having or developing asthma (*e.g.* a bronchial biopsy) or homogenate thereof, sputum, i.e. nasal lavage fluid (NLF) and BronchoAlveolar Lavage (BAL), and individual cellular components such as neutrophils, eosinophils, CD4+ T lymphocytes, monocytes and macrophages, the last mentioned including alveolar macrophages purified from NLF and BAL, all as described and illustrated herein by way of non-limiting example. The methods and compositions of the present invention are useful for screening, diagnosis and prognosis of a living subject, but may also be used for postmortem diagnosis in a subject, for example, to identify family members of the subject who are at risk of developing the same disease.

## 5.1. Definitions

"Feature" refers to a spot detected in a 2D gel, and the term "Chronic Asthma - Associated Feature" (CAF) refers to a feature that is differentially present in a sample from a subject having chronic asthma compared with a sample from a subject free from chronic asthma. A feature or spot detected in a 2D gel is characterized by its isoelectric point (pI) and molecular weight (MW) as determined by 2D gel electrophoresis, particularly utilizing the Preferred Technology described herein. As used herein, a feature is "differentially present" in a first sample with respect to a second sample when a method for detecting the said feature (*e.g.*, 2D electrophoresis) gives a different signal when applied to the first and second samples. A CAF, (or a protein isoform, i.e. CAPI, as defined *infra*) is "increased" in the first sample with respect to the second if the method of detection indicates that the CAF, or CAPI is more abundant in the first sample than in the second sample, or if the CAF, or CAPI is detectable in the first sample and substantially undetectable in the second sample. Conversely, a CAF, or CAPI is "decreased" in the first sample with respect to the second if the method of detection indicates that the CAF, or CAPI is less abundant in the first sample than in the second sample or if the CAF, or CAPI is undetectable in the first sample and detectable in the second sample.

Particularly, the relative abundance of a feature in two samples is determined in reference to its normalized signal, in two steps. First, the signal obtained upon detecting the feature in a sample is normalized by reference to a suitable background parameter, *e.g.*, (a) to the total protein in the sample being analyzed (*e.g.*, total protein loaded onto a gel); (b) to an Expression Reference Feature (ERF) i.e., a feature whose abundance is substantially invariant, within the limits of variability of the Preferred Technology, in the population of subjects being examined, *e.g.* the ERFs disclosed below, or (c) more preferably to the total signal detected as the sum of each of all proteins in the sample.

Secondly, the normalized signal for the feature in one sample or sample set is compared with the normalized signal for the same feature in another sample or sample

set in order to identify features that are "differentially present" in the first sample (or sample set) with respect to the second.

"Fold change" includes "fold increase" and "fold decrease" and refers to the relative increase or decrease in abundance of a CAF or the relative increase or decrease in expression or activity of a polypeptide (*e.g.* a CAPI, as defined *infra.*) in a first sample or sample set compared to a second sample (or sample set). A CAF or polypeptide (e.g. a CAPI) fold change may be measured by any technique known to those of skill in the art, albeit the observed increase or decrease will vary depending upon the technique used. Preferably, fold change is determined herein as described in the Examples *infra.*

"Chronic Asthma-Associated Protein Isoform" (CAPI) refers to a protein that is differentially present in a sample from a subject having chronic asthma compared with a sample from a subject free from chronic asthma or that is differentially present in a sample from a subject having one or more particular chronic asthma compared with a sample from a subject free from one or more particular chronic asthma. As used herein, a CAPI is "differentially present" in a first sample with respect to a second sample when a method for detecting the said feature, (*e.g.*, 2D electrophoresis or immunoassay) gives a different signal when applied to the first and second samples (refer to CAF definition).

A CAPI is characterised by one or more peptide sequences of which it is comprised, and further by a pI and MW, preferably determined by 2D electrophoresis, particularly utilising the Preferred Technology as described herein. Typically, CAPIs are identified or characterized by the amino acid sequencing of CAFs (Figure 1).

Figure 1 is a flow chart depicting the characterization of a CAF and relationship of a CAF and CAPI. A CAF may be further characterized as or by a CAPI having a particular peptide sequence associated with its pI and MW. As depicted herein, a CAF may comprise one or more CAPI(s), which have indistinguishable pI and MWs using the Preferred Technology, but which comprise distinct peptide sequences. The peptide sequence(s) of the CAPI can be utilized to search database(s) for previously identified proteins comprising such peptide

sequence(s). In some instances, it can be ascertained whether a commercially available antibody exists which may recognize the previously identified protein and/or a variant thereof. It should be noted that the CAPI may either correspond to the previously identified protein, or be a variant of the previously identified protein.

"Variant" as used herein refers to a polypeptide which is a member of a family of polypeptides that are encoded by a single gene or from a gene sequence within a family of related genes and which differ in their pI or MW, or both. Such variants can differ in their amino acid composition (*e.g.* as a result of alternative mRNA or premRNA processing, *e.g.* alternative splicing or limited proteolysis) and in addition, or in the alternative, may arise from differential post-translational modification (*e.g.*, glycosylation, acylation, phosphorylation).

"Modulate" in reference to expression or activity of a CAF, CAPI or a CAPI-related polypeptide refers to any change, *e.g.*, upregulation or downregulation, of the expression or activity of the CAF, CAPI or a CAPI-related polypeptide. Those skilled in the art, based on the present disclosure, will understand that such modulation can be determined by assays known to those of skill in the art.

"CAPI analog" refers to a polypeptide that possesses similar or identical function(s) as a CAPI but need not necessarily comprise an amino acid sequence that is similar or identical to the amino acid sequence of the CAPI, or possess a structure that is similar or identical to that of the CAPI. As used herein, an amino acid sequence of a polypeptide is "similar" to that of a CAPI if it satisfies at least one of the following criteria: (a) the polypeptide has an amino acid sequence that is at least 30% (more preferably, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99%) identical to the amino acid sequence of the CAPI; (b) the polypeptide is encoded by a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence encoding at least 5 amino acid residues (more preferably, at least 10 amino acid residues, at least 15 amino acid residues, at least 20 amino acid residues, at least 25 amino acid residues, at least 40 amino acid residues, at least 50 amino acid residues, at least 60 amino residues, at least 70 amino

acid residues, at least 80 amino acid residues, at least 90 amino acid residues, at least 100 amino acid residues, at least 125 amino acid residues, or at least 150 amino acid residues) of the CAPI; or (c) the polypeptide is encoded by a nucleotide sequence that is at least 30% (more preferably, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99%) identical to the nucleotide sequence encoding the CAPI. As used herein, a polypeptide with "similar structure" to that of a CAPI refers to a polypeptide that has a similar secondary, tertiary or quarternary structure as that of the CAPI. The structure of a polypeptide can determined by methods known to those skilled in the art, including but not limited to, X-ray crystallography, nuclear magnetic resonance, and crystallographic electron microscopy.

"CAPI fusion protein" refers to a polypeptide that comprises (i) an amino acid sequence of a CAPI, a CAPI fragment, a CAPI-related polypeptide or a fragment of a CAPI-related polypeptide and (ii) an amino acid sequence of a heterologous polypeptide (i.e., a non-CAPI, non-CAPI fragment or non-CAPI-related polypeptide).

"CAPI homolog" refers to a polypeptide that comprises an amino acid sequence similar to that of a CAPI but does not necessarily possess a similar or identical function as the CAPI.

"CAPI ortholog" refers to a non-human polypeptide that (i) comprises an amino acid sequence similar to that of a CAPI and (ii) possesses a similar or identical function to that of the CAPI.

"CAPI-related polypeptide" refers to a CAPI homolog, a CAPI analog, a variant of CAPI, a CAPI ortholog, or any combination thereof.

"Chimeric Antibody" refers to a molecule in which different portions are derived from different animal species, such as those having a human immunoglobulin constant region and a variable region derived from a murine mAb. (See, *e.g.*, Cabilly et al., U.S. Patent No. 4,816,567; and Boss et al., U.S. Patent No. 4,816397, which are incorporated herein by reference in their entirety.)

"Derivative" refers to a polypeptide that comprises an amino acid sequence of a second polypeptide that has been altered by the introduction of amino acid residue substitutions, deletions or additions. The derivative polypeptide possesses a similar or identical function as the second polypeptide.

"Fragment" refers to a peptide or polypeptide comprising an amino acid sequence of at least 5 amino acid residues (preferably, at least 10 amino acid residues, at least 15 amino acid residues, at least 20 amino acid residues, at least 25 amino acid residues, at least 40 amino acid residues, at least 50 amino acid residues, at least 60 amino residues, at least 70 amino acid residues, at least 80 amino acid residues, at least 90 amino acid residues, at least 100 amino acid residues, at least 125 amino acid residues, at least 150 amino acid residues, at least 175 amino acid residues, at least 200 amino acid residues, or at least 250 amino acid residues) of the amino acid sequence of a second polypeptide. The fragment of a CAPI may or may not possess a functional activity of the second polypeptide.

The "percent identity" of two amino acid sequences or of two nucleic acid sequences can be or is generally determined by aligning the sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in either sequence for best alignment with the other sequence) and comparing the amino acid residues or nucleotides at corresponding positions. The "best alignment" is an alignment of two sequences that results in the highest percent identity. The percent identity is determined by the number of identical amino acid residues or nucleotides in the sequences being compared (i.e., % identity = # of identical positions/total # of positions x 100).

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm known to those of skill in the art. An example of a mathematical algorithm for comparing two sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264-2268, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877. The NBLAST and XBLAST programs of Altschul, et al. (1990) J. Mol. Biol. 215:403-410 have incorporated such an algorithm. BLAST nucleotide searches can be performed with

the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (1997) Nucleic Acids Res. 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (*Id.*). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

Another example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). The ALIGN program (version 2.0) which is part of the GCG sequence alignment software package has incorporated such an algorithm. Other algorithms for sequence analysis known in the art include ADVANCE and ADAM as described in Torellis and Robotti (1994) Comput. Appl. Biosci., 10 :3-5; and FASTA described in Pearson and Lipman (1988) Proc. Natl. Acad. Sci. 85:2444-8. Within FASTA, ktup is a control option that sets the sensitivity and speed of the search.

"Diagnosis" refers to diagnosis, prognosis, monitoring, characterizing, selecting patients, including participants in clinical trials, and identifying patients at risk for or having a particular disorder or those most likely to respond to a particular therapeutic treatment, or for assessing or monitoring a patient's response to a particular therapeutic treatment.

"Treatment" refers to therapy, prevention and prophylaxis and particularly refers to the administration of medicine or the performance of medical procedures with respect to a patient, for either prophylaxis (prevention) or to cure the infirmity or malady in the instance where the patient is afflicted.

"Agent" refers to all materials that may be used to prepare pharmaceutical and diagnostic compositions, or that may be compounds, nucleic acids, polypeptides,

fragments, isoforms, variants, or other materials that may be used independently for such purposes, all in accordance with the present invention.

## 5.2. Chronic Asthma-Associated Features (CAFs)

In one aspect of the invention, two-dimensional electrophoresis is used to analyze tissue from a subject, preferably a living subject, in order to detect or quantify the expression of one or more Chronic Asthma-Associated Features (CAFs) for screening, prevention or diagnosis of chronic asthma, to determine the prognosis of a subject having chronic asthma, to monitor progression of chronic asthma, to monitor the effectiveness of chronic asthma therapy, for identifying patients most likely to respond to a particular therapeutic treatment, or for drug development.

By way of example and not of limitation, using the Preferred Technology, a number of samples from primary cultures of human airway myofibroblasts subjected to stimulation with thrombin and/or treatment with dexamethasone or theophylline, and their respective controls as described in the Examples, *infra*, are separated by two-dimensional electrophoresis, and the fluorescent digital images of the resulting gels are matched to a chosen representative primary master gel image. This process allows any gel feature, characterised by its pI and MW, to be identified and examined on any gel of the study. In particular, the amount of protein present in a given feature can be measured in each gel; this feature abundance can be averaged amongst gels from similar samples (e.g. gels from samples of primary cultures having thrombin treatment). Finally, statistical analyses can be conducted on the thus created sample sets, in order to compare 2 or more sample sets to each other.

As used herein, "two-dimensional electrophoresis" (2D-electrophoresis) means a technique comprising isoelectric focusing, followed by denaturing electrophoresis; this generates a two-dimensional gel (2D-gel) containing a plurality of separated proteins. Preferably, the step of denaturing electrophoresis uses polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Especially preferred are the highly accurate and automatable methods and

apparatus ("the Preferred Technology") described in International Application No. 97GB3307, filed December 1, 1997, (published as WO 98/23950) and in U.S. Patent No. 6,064,754, each of which is incorporated herein by reference in its entirety with particular reference to the protocol at pages 23-35. Briefly, the Preferred Technology provides efficient, computer-assisted methods and apparatus for identifying, selecting and characterizing biomolecules (*e.g.* proteins, including glycoproteins) in a biological sample. A two-dimensional array is generated by separating biomolecules on a two-dimensional gel according to their electrophoretic mobility and isoelectric point. A computer-generated digital profile of the array is generated, representing the identity, apparent molecular weight, isoelectric point, and relative abundance of a plurality of biomolecules detected in the two-dimensional array, thereby permitting computer-mediated comparison of profiles from multiple biological samples, as well as computer aided excision of separated proteins of interest.

A preferred scanner for detecting fluorescently labelled proteins is described in WO 96/36882 and in the Ph.D. thesis of David A. Basiji, entitled "Development of a High-throughput Fluorescence Scanner Employing Internal Reflection Optics and Phase-sensitive Detection (Total Internal Reflection, Electrophoresis)", University of Washington (1997), Volume 58/12-B of Dissertation Abstracts International, page 6686, the contents of each of which are incorporated herein by reference. These documents describe an image scanner designed specifically for automated, integrated operation at high speeds. The scanner can image gels that have been stained with fluorescent dyes or silver stains, as well as storage phosphor screens. The Basiji thesis provides a phase-sensitive detection system for discriminating modulated fluorescence from baseline noise due to laser scatter or homogeneous fluorescence, but the scanner can also be operated in a non-phase-sensitive mode. This phase-sensitive detection capability would increase the sensitivity of the instrument by an order of magnitude or more compared to conventional fluorescence imaging systems. The increased sensitivity would reduce the sample-preparation load on the upstream instruments while the enhanced image quality simplifies image analysis downstream in the process.

A more highly preferred scanner is the Apollo 2 scanner (Oxford Glycosciences, Oxford, UK), which is a modified version of the above described scanner. In the Apollo 2 scanner, the gel is transported through the scanner on a precision lead-screw drive system. This is preferable to laying the glass plate on the belt-driven system that is described in the Basiji thesis, as it provides a reproducible means of accurately transporting the gel past the imaging optics.

In the Apollo 2 scanner, the gel is secured against three alignment stops that rigidly hold the glass plate in a known position. By doing this in conjunction with the above precision transport system, the absolute position of the gel can be predicted and recorded. This ensures that co-ordinates of each feature on the gel can be determined more accurately and communicated, if desired, to a cutting robot for excision of the feature. In the Apollo 2 scanner, the carrier that holds the gel has four integral fluorescent markers for use to correct the image geometry. These markers are a quality control feature that confirms that the scanning has been performed correctly.

In comparison to the scanner described in the Basiji thesis, the optical components of the Apollo 2 scanner have been inverted. In the Apollo 2 scanner, the laser, mirror, waveguide and other optical components are above the glass plate being scanned. The scanner described in the Basiji thesis has these components underneath. In the Apollo 2 scanner, the glass plate is mounted onto the scanner gel side down, so that the optical path remains through the glass plate. By doing this, any particles of gel that may break away from the glass plate will fall onto the base of the instrument rather than into the optics. This does not affect the functionality of the system, but increases its reliability.

Still more preferred is the Apollo 3 scanner, in which the signal output is digitized to the full 16-bit data without any peak saturation or without square root encoding of the signal. A compensation algorithm has also been applied to correct for any variation in detection sensitivity along the path of the scanning beam. This variation is due to anomalies in the optics and differences in collection efficiency across the waveguide. A calibration is performed using a perspex plate with an even fluorescence throughout. The data received from a scan of this plate are used to

determine the multiplication factors needed to increase the signal from each pixel level to a target level. These factors are then used in subsequent scans of gels to remove any internal optical variations.

As defined above, the term "feature" refers to a spot detected in a 2D gel, and the term "Chronic Asthma-Associated Feature" (CAF) refers to a feature that is differentially present in a sample (*e.g.* a bronchial biopsy) from a subject having chronic asthma compared with a sample (*e.g.* a bronchial biopsy) from a subject free from chronic asthma.

In accordance with an aspect of the present invention, the CAFs disclosed herein have been identified by comparing primary cultures of human airway myofibroblasts subjected to stimulation with thrombin and/or treatment with dexamethasone or theophylline, against their respective controls as described in the Examples, *infra*. The relevance of primary cultures of human airway myofibroblasts to the study of chronic asthma, and the use of thrombin to promote inflammatory response have been largely covered in the literature:

- Hall IP, Kotlikoff M, *Am J Physiol* 1995 Jan;268(1 Pt 1):L1-11, Use of cultured airway myocytes for study of airway smooth muscle.
- Billington CK, Joseph SK, Swan C, Scott MG, Jobson TM, Hall IP, *Am J Physiol* 1999 Mar;276(3 Pt 1):L412-9, Modulation of human airway smooth muscle proliferation by type 3 phosphodiesterase inhibition.
- Panettieri RA Jr, Hall IP, Maki CS, Murray RK, *Am J Respir Cell Mol Biol* 1995 Aug;13(2):205-16, Alpha-Thrombin increases cytosolic calcium and induces human airway smooth muscle cell proliferation.
- Hirst SJ, *Eur Respir J* 1996 Apr;9(4):808-20, Airway smooth muscle cell culture: application to studies of airway wall remodelling and phenotype plasticity in asthma.

The CAFs identified through the methods and apparatus of the Preferred Technology are listed in Table I. Six different experiments were run with their

respective controls, and for each experiment Table I indicates for which conditions the CAFs were found to be increased or decreased.

Table I

Table I

| CAF # | pI | MW | Condition |
|---|---|---|---|
| CAF-1 | 5.37 | 122123 | 2, 3, |
| CAF-2 | 5.16 | 119778 | 1, 3, |
| CAF-3 | 5.3 | 116172 | 2, 3, |
| CAF-5 | 5.02 | 108632 | 2, 4, |
| CAF-12 | 6.55 | 61580 | 2, |
| CAF-13 | 5.62 | 60360 | 2, 3, |
| CAF-15 | 5.28 | 55571 | 2, 3, |
| CAF-18 | 7.67 | 35754 | 2, 3, |
| CAF-19 | 7.91 | 34254 | 4, |
| CAF-20 | 5.07 | 30903 | 2, |
| CAF-21 | 7.68 | 28655 | 2, 3, |
| CAF-22 | 5.27 | 26368 | 1 |
| CAF-24 | 5.23 | 49444 | 2, |
| CAF-25 | 5.62 | 37110 | 2, 4, |
| CAF-29 | 7.46 | 60270 | 1, 3, |
| CAF-33 | 4.88 | 40521 | 4, |
| CAF-34 | 6.47 | 33980 | 1, 3, |
| CAF-35 | 8.16 | 22061 | 4, |

1 – Increased in early response to inflammation, 2 – Decreased in early response to inflammation, 3 – Increased in late response to inflammation, 4 – Decreased in late response to inflammation.

For any given CAF, the signal obtained upon analyzing tissue from subjects having chronic asthma relative to the signal obtained upon analyzing tissue from subjects free from chronic asthma will depend upon the particular analytical protocol

and detection technique that is used. Accordingly, those skilled in the art will understand that any laboratory, based on the present description, can establish a suitable reference range for any CAF in subjects free from chronic asthma according to the analytical protocol and detection technique in use, as is conventional in the diagnostic art. Preferably, at least one positive control tissue sample from a subject known to have chronic asthma or at least one negative control tissue sample from a subject known to be free from chronic asthma (and more preferably both positive and negative control samples) are included in each batch of test samples analyzed. In one embodiment, the level of expression of a feature is determined relative to a background value, which is defined as the level of signal obtained from a proximal region of the image that (a) is equivalent in area to the particular feature in question; and (b) contains no discernable protein feature.

In a preferred embodiment, the signal associated with a CAF in the tissue of a subject (*e.g.*, a subject suspected of having or known to have chronic asthma) is normalized with reference to one or more ERFs detected in the same 2D gel. As will be apparent to one of ordinary skill in the art, such ERFs may readily be determined by comparing different samples using the Preferred Technology. Suitable ERFs include (but are not limited to) that described in the following table.

Table II. Expression Reference Features Identified in this Study

| ERF # | pI | MW (D) |
|---|---|---|
| ERF-1 | 5.72 | 57517 |
| ERF-2 | 5.60 | 31474 |
| ERF-3 | 5.60 | 26491 |

As those of skill in the art will readily appreciate, the measured MW and pI of a given feature or protein isoform will vary to some extent depending on the precise protocol used for each step of the 2D electrophoresis and for landmark matching. As used herein, the terms "MW" and "pI" are defined, respectively, to mean the apparent

molecular weight in Daltons and the apparent isoelectric point of a feature or protein isoform as measured in exact accordance with the Reference Protocol identified in Section 6 below. When the Reference Protocol is followed and when samples are run in duplicate or a higher number of replicates, variation in the measured mean pI of a CAF or CAPI is typically less than 3% and variation in the measured mean MW of a CAF or CAPI is typically less than 5%. Where the skilled artisan wishes to deviate from the Reference Protocol, calibration experiments should be performed to compare the MW and pI for each CAF or protein isoform as detected (a) by the Reference Protocol and (b) by the deviant protocol.

CAFs modulated in the presence of an early inflammatory asthmatic response

CAFs can be used for detection, prognosis, diagnosis, or monitoring of chronic asthma or for drug development. In one embodiment of the invention, a bronchial biopsy (*e.g.*, from a subject suspected of having chronic asthma, for definition see *infra*) is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CAFs: CAF-2, CAF-22, CAF-29 or CAF-34. An increased abundance of said one or more CAFs in the tissue from the subject relative to tissue from a subject or subjects free from chronic asthma (*e.g.*, a control sample or a previously determined reference range) indicates the presence of an early inflammatory asthmatic response.

More preferably a bronchial biopsy (*e.g.*, a subject suspected of having chronic asthma) is analyzed by 2D electrophoresis for quantitative detection of the following CAF: CAF-22, whose increased expression in the early inflammatory asthmatic response can be returned (at least partially) to basal levels by the administration of asthma-related therapies.

In another embodiment of the invention, a bronchial biopsy (*e.g.*, a subject suspected of having chronic asthma) is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CAFs: CAF-1, CAF-3, CAF-5, CAF-12, CAF-13, CAF-15, CAF-18, CAF-20, CAF-21, CAF-24, CAF-25. A decreased abundance of said one or more CAFs in the tissue from the subject relative to tissue

from a subject or subjects free from chronic asthma (*e.g.*, a control sample or a previously determined reference range) indicates the presence of an early inflammatory asthmatic response. More preferably a bronchial biopsy (*e.g.*, a subject suspected of having chronic asthma) is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CAFs: CAF-1, CAF-3, CAF-12, CAF-13, CAF-15, CAF-18, CAF-20, CAF-24, CAF-25 whose decreased expression in the early inflammatory asthmatic response can be returned (at least partially) to basal levels by the administration of asthma-related therapies.

In yet another embodiment, a bronchial biopsy is analyzed by 2D electrophoresis for quantitative detection of (a) one or more CAFs or any combination of them, whose increased abundance indicates the presence of an early inflammatory asthmatic response, *i.e.*, CAFs: CAF-2, CAF-22, CAF-29 and CAF-34, more preferably CAF-22, and/or (b) one or more CAFs or any combination of them, whose decreased abundance indicates the presence of an early inflammatory asthmatic response *i.e.*, CAFs: CAF-1, CAF-3, CAF-5, CAF-12, CAF-13, CAF-15, CAF-18, CAF-20, CAF-21, CAF-24, CAF-25, more preferably CAFs: CAF-1, CAF-3, CAF-12, CAF-13, CAF-15, CAF-18, CAF-20, CAF-24, CAF-25.

In yet another embodiment of the invention, a bronchial biopsy is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CAFs: CAF-1, CAF-2, CAF-3, CAF-5, CAF-12, CAF-13, CAF-15, CAF-18, CAF-20, CAF-21, CAF-22, CAF-24, CAF-25, CAF-29, CAF-34, wherein the ratio of the one or more CAFs relative to an Expression Reference Feature (ERF) indicates whether an early inflammatory asthmatic response is present. More preferably a bronchial biopsy is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CAFs: CAF-1, CAF-3, CAF-12, CAF-13, CAF-15, CAF-18, CAF-20, CAF-24, CAF-25, where the ratio of the one or more CAFs relative to an Expression Reference Feature (ERF) indicates whether an early inflammatory asthmatic response which is responsive to treatment by asthma-related therapies is present.

In a specific embodiment, an increase in one or more CAF/ERF ratios in a test sample relative to the CAF/ERF ratios in a control sample or a reference range

indicates the presence of an early inflammatory asthmatic response; CAF-2, CAF-22, CAF-29 and CAF-34 are suitable CAFs for this purpose. More preferably, an increase in one or more CAF/ERF ratios in a test sample relative to the CAF/ERF ratios in a control sample or a reference range indicates the presence of an early inflammatory asthmatic response which is responsive to asthma-related therapies; CAF-22 is a suitable CAF for this purpose.

In another specific embodiment, a decrease in one or more CAF/ERF ratios in a test sample relative to the CAF/ERF ratios in a control sample or a reference range indicates the presence of an early inflammatory asthmatic response; CAF-1, CAF-3, CAF-5, CAF-12, CAF-13, CAF-15, CAF-18, CAF-20, CAF-21, CAF-24, CAF-25, are suitable CAFs for this purpose. More preferably, a decrease in one or more CAF/ERF ratios in a test sample relative to the CAF/ERF ratios in a control sample or a reference range indicates the presence of an early inflammatory asthmatic response which is responsive to asthma-related therapies; CAF-1, CAF-3, CAF-12, CAF-13, CAF-15, CAF-18, CAF-20, CAF-24, CAF-25, are suitable CAFs for this purpose.

In a further embodiment of the invention, a bronchial biopsy is analyzed by 2D electrophoresis for quantitative detection of (a) one or more CAFs, or any combination of them, whose increased CAF/ERF ratio(s) in a test sample relative to the CAF/ERF ratio(s) in a control sample indicates the presence of an early inflammatory asthmatic response, *i.e.*, CAF-2, CAF-22, CAF-29 and CAF-34, more preferably CAF-22, or (b) one or more CAFs, or any combination of them, whose decreased CAF/ERF ratio(s) in a test sample relative to the CAF/ERF ratio(s) in a control sample indicates the presence of an early inflammatory asthmatic response, *i.e.*, CAF-1, CAF-3, CAF-5, CAF-12, CAF-13, CAF-15, CAF-18, CAF-20, CAF-21, CAF-24, CAF-25, more preferably CAF-1, CAF-3, CAF-12, CAF-13, CAF-15, CAF-18, CAF-20, CAF-24 or CAF-25.

<u>CAFs modulated in the presence of a late inflammatory asthmatic response</u>

In a further embodiment of the invention, a bronchial biopsy (*e.g.*, from a subject suspected of having chronic asthma, for definition see *infra*) is analyzed by 2D

electrophoresis for quantitative detection of one or more of the following CAFs: CAF-1, CAF-2, CAF-3, CAF-13, CAF-15, CAF-18, CAF-21, CAF-29, or CAF-34. An increased abundance of said one or more CAFs in the tissue from the subject relative to tissue from a subject or subjects free from chronic asthma (*e.g.*, a control sample or a previously determined reference range) indicates the presence of a late inflammatory asthmatic response. More preferably a bronchial biopsy (*e.g.*, a subject suspected of having chronic asthma) is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CAFs: CAF-1, CAF-15 or CAF-29, whose increased expression in the late inflammatory asthmatic response can be returned (at least partially) to basal levels by the administration of asthma-related therapies.

CAFs can be used for detection, prognosis, diagnosis, or monitoring of chronic asthma or for drug development. In one embodiment of the invention, a bronchial biopsy (*e.g.*, a subject suspected of having chronic asthma) is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CAFs: CAF-5, CAF-19, CAF-25, CAF-33 or CAF-35. A decreased abundance of said one or more CAFs in the tissue from the subject relative to tissue from a subject or subjects free from chronic asthma (*e.g.*, a control sample or a previously determined reference range) indicates the presence of a late inflammatory asthmatic response. More preferably a bronchial biopsy (*e.g.*, a subject suspected of having chronic asthma) is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CAFs: CAF-19, CAF-25, CAF-33 or CAF-35 whose decreased expression in the late inflammatory asthmatic response can be returned (at least partially) to basal levels by the administration of asthma-related therapies.

In yet another embodiment, a bronchial biopsy is analyzed by 2D electrophoresis for quantitative detection of (a) one or more CAFs or any combination of them, whose increased abundance indicates the presence of a late inflammatory asthmatic response, *i.e.*, CAFs: CAF-1, CAF-2, CAF-3, CAF-13, CAF-15, CAF-18, CAF-21, CAF-29, or CAF-34, more preferably CAF-1, CAF-15 or CAF-29, and (b) one or more CAFs or any combination of them, whose decreased abundance indicates the presence of a late inflammatory asthmatic response *i.e.*, CAFs: CAF-5, CAF-19,

CAF-25, CAF-33 or CAF-35 more preferably CAFs: CAF-19, CAF-25, CAF-33 or CAF-35.

In yet another embodiment of the invention, a bronchial biopsy is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CAFs: CAF-1, CAF-2, CAF-3, CAF-13, CAF-15, CAF-18, CAF-21, CAF-29, or CAF-34, wherein the ratio of the one or more CAFs relative to an Expression Reference Feature (ERF) indicates whether a late inflammatory asthmatic response is present. More preferably a bronchial biopsy is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CAFs: CAF-1, CAF-5, CAF-15, CAF-19, CAF-25, CAF-29, CAF-33 or CAF-35 where the ratio of the one or more CAFs relative to an Expression Reference Feature (ERF) indicates whether a late inflammatory asthmatic response which is responsive to treatment by asthma-related therapies is present. In a specific embodiment, an increase in one or more CAF/ERF ratios in a test sample relative to the CAF/ERF ratios in a control sample or a reference range indicates the presence of a late inflammatory asthmatic response; CAF-1, CAF-2, CAF-3, CAF-13, CAF-15, CAF-18, CAF-21, CAF-29, or CAF-34 are suitable CAFs for this purpose. More preferably, an increase in one or more CAF/ERF ratios in a test sample relative to the CAF/ERF ratios in a control sample or a reference range indicates the presence of a late inflammatory asthmatic response which is responsive to asthma-related therapies; CAF-1, CAF-15 or CAF-29 are suitable CAFs for this purpose. In another specific embodiment, a decrease in one or more CAF/ERF ratios in a test sample relative to the CAF/ERF ratios in a control sample or a reference range indicates the presence of a late inflammatory asthmatic response; CAF-5, CAF-19, CAF-25, CAF-33 or CAF-35 are suitable CAFs for this purpose. More preferably, a decrease in one or more CAF/ERF ratios in a test sample relative to the CAF/ERF ratios in a control sample or a reference range indicates the presence of a late inflammatory asthmatic response which is responsive to asthma-related therapies; CAF-19, CAF-25, CAF-33 or CAF-35 are suitable CAFs for this purpose.

In a further embodiment of the invention, a bronchial biopsy is analyzed by 2D electrophoresis for quantitative detection of (a) one or more CAFs, or any

combination of them, whose increased CAF/ERF ratio(s) in a test sample relative to the CAF/ERF ratio(s) in a control sample indicates the presence of a late inflammatory asthmatic response, *i.e.*, CAF-1, CAF-2, CAF-3, CAF-13, CAF-15, CAF-18, CAF-21, CAF-29, or CAF-34, more preferably CAF-1, CAF-15 or CAF-29; (b) one or more CAFs, or any combination of them, whose decreased CAF/ERF ratio(s) in a test sample relative to the CAF/ERF ratio(s) in a control sample indicates the presence of a late inflammatory asthmatic response, *i.e.*, CAF-5, CAF-19, CAF-25, CAF-33 or CAF-35, more preferably CAF-19, CAF-25, CAF-33 or CAF-35.

In a preferred embodiment, a bronchial biopsy from a subject is analyzed for quantitative detection of a plurality of CAFs.

## 5.3. Chronic Asthma-Associated Protein Isoforms (CAPIs)

In another aspect of the invention, tissue from a subject, preferably a living subject, is analyzed for quantitative detection of one or more Chronic Asthma-Associated Protein Isoforms (CAPIs) for screening or diagnosis of chronic asthma, to determine the prognosis of a subject having chronic asthma, to monitor the effectiveness of chronic asthma therapy, for identifying patients most likely to respond to a particular therapeutic treatment or for drug development. As is well known in the art, a given protein may be expressed as one or more variant forms that differ in amino acid composition (*e.g.*, as a result of alternative mRNA or premRNA processing, *e.g.* alternative splicing or limited proteolysis) or as a result of differential post-translational modification (*e.g.*, glycosylation, phosphorylation, acylation), or both, so that proteins of identical amino acid sequence can differ in their pI, MW, or both. "Chronic Asthma -Associated Protein Isoform" refers to a protein isoform that is differentially present in tissue from a subject having chronic asthma compared with tissue from a subject free from chronic asthma.

CAPIs have been identified by partial amino acid sequencing of CAFs, using the methods and apparatus of the Preferred Technology. One skilled in the art can identify sequence information from proteins analyzed by mass spectrometry and/or tandem mass spectrometry using various spectral interpretation methods and database

searching tools. Examples of some of these methods and tools can be found at the Swiss Institute of Bioinformatics web site at http://www.expasy.ch/, and the European Molecular Biology Laboratory web site at www.mann.embl-heidelberg.de/Services/PeptideSearch/. Identification of CAPIs was performed primarily using the SEQUEST search program (Eng et al., 1994, J. Am. Soc. Mass Spectrom. 5:976-989) and the method described in PCT Application No. PCT/GB01/04034, which is incorporated herein by reference in its entirety.

The CAPIs are listed in Table III. The amino acid sequences of tryptic digest peptides of these CAPIs identified by tandem mass spectrometry and database searching as described in the Examples, *infra* are listed Table IV, in addition to their corresponding pIs and MWs.

Table III: CAPIs desciption by apparent molecular weight (MW) and isoelectric point (pI)

Table III

| CAF# | CAPI | pI | MW (Da) | Condition |
|---|---|---|---|---|
| CAF-1 | CAPI-1 | 5.37 | 122123 | 2, 3 |
| CAF-2 | CAPI-2 | 5.16 | 119778 | 1, 3 |
| CAF-3 | CAPI-3 | 5.30 | 116172 | 2, 3 |
| CAF-5 | CAPI-5 | 5.02 | 108632 | 2, 4 |
| CAF-12 | CAPI-12 | 6.55 | 61580 | 2 |
| CAF-13 | CAPI-13 | 5.62 | 60360 | 2, 3 |
| CAF-15 | CAPI-15 | 5.28 | 55571 | 2, 3 |
| CAF-18 | CAPI-18 | 7.67 | 35754 | 2, 3 |
| CAF-19 | CAPI-19 | 7.91 | 34254 | 4 |
| CAF-20 | CAPI-20 | 5.07 | 30903 | 2 |
| CAF-21 | CAPI-21 | 7.68 | 28655 | 2, 3 |
| CAF-22 | CAPI-22 | 5.27 | 26368 | 1 |
| CAF-24 | CAPI-24 | 5.23 | 49444 | 2 |

Table III

| CAF # | CAPI # | pI | MW (Da) | Conditions |
|---|---|---|---|---|
| CAF-25 | CAPI-25 | 5.62 | 37110 | 2, 4 |
| CAF-29 | CAPI-29 | 7.46 | 60270 | 1, 3 |
| CAF-29 | CAPI-30 | 7.46 | 60270 | 1, 3 |
| CAF-33 | CAPI-34 | 4.88 | 40521 | 4 |
| CAF-34 | CAPI-35 | 6.47 | 33980 | 1, 3 |
| CAF-35 | CAPI-36 | 8.16 | 22061 | 4 |
| CAF-13 | CAPI-37 | 5.62 | 60360 | 2 |
| CAF-20 | CAPI-38 | 5.07 | 30903 | 2 |

1 – Increased in early response to inflammation, 2 – Decreased in early response to inflammation, 3 – Increased in late response to inflammation, 4 – Decreased in late response to inflammation.

Table IV: CAPIs description by partial sequence

**Table IV**

| CAF # | CAPI # | Amino Acid Sequence of Tryptic Digest Peptides | SEQ ID NO |
|---|---|---|---|
| CAF-1 | CAPI-1 | TINEVENQILTR | SEQ ID NO: 40 |
| CAF-2 | CAPI-2 | AGTQIENIEEDFR<br>TINEVENQILTR | SEQ ID NO: 4,<br>SEQ ID NO: 40 |
| CAF-3 | CAPI-3 | AGTQIENIDEDFR<br>GYEEWLLNEIR<br>HRPELIEYDK | SEQ ID NO: 3,<br>SEQ ID NO: 21,<br>SEQ ID NO: 22 |
| CAF-5 | CAPI-5 | GVVDSEDLPLNISR | SEQ ID NO: 20 |
| CAF-12 | CAPI-12 | ADNFEYSDPVDGSISR<br>FNISNGGPAPEAITDK | SEQ ID NO: 2,<br>SEQ ID NO: 15 |
| CAF-13 | CAPI-13 | SPLGEVAIR<br>VVLFEMEAR | SEQ ID NO: 37,<br>SEQ ID NO: 47 |
| CAF-13 | CAPI-37 | SPLGEVAIR<br>VVLFEMEAR | SEQ ID NO: 37,<br>SEQ ID NO: 47 |

**Table IV**

| CAF# | CAPI# | Amino Acid Sequence of Tryptic Digest Peptides | SEQ ID NO: |
|---|---|---|---|
| CAF-15 | CAPI-15 | DPLELFR | SEQ ID NO: 8, |
| | | ELTGEDVLVR | SEQ ID NO: 12, |
| | | LEAPLEELR | SEQ ID NO: 27 |
| CAF-18 | CAPI-18 | TNQELQEINR | SEQ ID NO: 41 |
| CAF-19 | CAPI-19 | GALQNIIPASTGAAK | SEQ ID NO: 16, |
| | | LISWYDNEFGYSNR | SEQ ID NO: 28, |
| | | LVINGNPITIFQER | SEQ ID NO: 30 |
| CAF-20 | CAPI-20 | VEEEIVTLR | SEQ ID NO: 43 |
| CAF-20 | CAPI-38 | GLLSDSMTDVPVDTGVAAR | SEQ ID NO: 17, |
| | | SWHDVQVSSAYVK | SEQ ID NO: 38, |
| | | VTQSDLYK | SEQ ID NO: 46, |
| | | VEEEIVTLR | SEQ ID NO: 43 |
| CAF-21 | CAPI-21 | EALTYDGALLGDR | SEQ ID NO: 10 |
| CAF-22 | CAPI-22 | QEAVALLQGQR | SEQ ID NO: 35 |
| CAF-24 | CAPI-24 | SYELPDGQVITIGNER | SEQ ID NO: 39 |
| CAF-25 | CAPI-25 | GNVGFVFTK | SEQ ID NO: 19, |
| | | IIQLLDDYPK | SEQ ID NO: 24, |
| | | VLALSVETDYTFPLAEK | SEQ ID NO: 45 |
| CAF-29 | CAPI-29 | AIFLADGNVFTTGFSR | SEQ ID NO: 5, |
| | | QLALWNPK | SEQ ID NO: 36, |
| | | VGIVAWHPTAR | SEQ ID NO: 44 |
| CAF-29 | CAPI-30 | DFAAEVVHPGDLK | SEQ ID NO: 6, |
| | | EYTLDVYR | SEQ ID NO: 14, |
| | | NSEPEEVIPSR | SEQ ID NO: 34 |
| CAF-33 | CAPI-34 | DISTNYYASQK | SEQ ID NO: 7, |
| | | EAESSPFVER | SEQ ID NO: 9, |

**Table IV**

| CAF # | CAPI # | Amino Acid Sequence of Tryptic Digest Peptides | SEQ ID NO |
|---|---|---|---|
| | | EFEPLLNWMK | SEQ ID NO: 11, |
| | | IADDKYNDTFWK | SEQ ID NO: 23, |
| | | KEAESSPFVER | SEQ ID NO: 25, |
| | | KTFEINPR | SEQ ID NO: 26, |
| | | LSLNIDPDAK | SEQ ID NO: 29, |
| | | YNDTFWK | SEQ ID NO: 48 |
| CAF-34 | CAPI-35 | ADEDPALFQSVK | SEQ ID NO: 1, |
| | | TVEIVHIDIADR | SEQ ID NO: 42 |
| CAF-35 | CAPI-36 | EWHHFLVVNMK | SEQ ID NO: 13, |
| | | GNDISSGTVLSDYVGSGPPK | SEQ ID NO: 18, |
| | | LYEQLSGK | SEQ ID NO: 31, |
| | | LYTLVLTDPDAPSR | SEQ ID NO: 32, |
| | | NRPTSISWDGLDSGK | SEQ ID NO: 33, |
| | | YVWLVYEQDRPLK | SEQ ID NO: 49 |

As will be evident to one of skill in the art, based upon the present description, a given CAPI can be described according to the data provided for that CAPI in Tables III and IV. The CAPI is a protein comprising a peptide sequence described for that CAPI (preferably comprising a plurality of, more preferably all of, the peptide sequences described for that CAPI) and has a pI of about the value stated for that CAPI (preferably within about 10%, more preferably within about 5% still more preferably within about 1% of the stated value) and has a MW of about the value stated for that CAPI (preferably within about 10%, more preferably within about 5%, still more preferably within about 1% of the stated value).

CAPIs modulated in the presence of an early inflammatory asthmatic response

In one embodiment, a bronchial biopsy is analyzed for quantitative detection of one or more of the following CAPIs: CAPI-2, CAPI-22, CAPI-29, CAPI-30 or CAPI-35 or any combination of them, wherein an increased abundance of the CAPI or CAPIs (or any combination of them) in the tissue from the subject relative to tissue from a subject or subjects free from chronic asthma (*e.g.*, a control sample or a previously determined reference range) indicates the presence of an early inflammatory asthmatic response. More preferably a bronchial biopsy (*e.g.*, from a subject suspected of having chronic asthma) is analyzed by 2D electrophoresis for quantitative detection of the following CAPI: CAPI-22, whose increased expression in the early inflammatory asthmatic response can be returned (at least partially) to basal levels by the administration of asthma-related therapies

In another embodiment of the invention, a bronchial biopsy is analyzed for quantitative detection of one or more of the following CAPIs: CAPI-1, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-20, CAPI-21, CAPI-24, CAPI-25, CAPI-37 or CAPI-38 whose decreased expression indicates the presence of an early inflammatory asthmatic response. More preferably a sample (*e.g.*, from a subject suspected of having chronic asthma) is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CAPIs: CAPI-1, CAPI-3, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-20, CAPI-24, CAPI-25, CAPI-37 or CAPI-38, whose decreased expression in the early inflammatory asthmatic response can be returned (at least partially) to basal levels by the administration of asthma-related therapies

In a further embodiment, a bronchial biopsy is analyzed for quantitative detection of (a) one or more CAPIs, or any combination of them, whose increased abundance indicates the presence of an early inflammatory asthmatic response, *i.e.*, CAPI-2, CAPI-22, CAPI-29, CAPI-30 or CAPI-35, more preferably CAPI-22 and (b) one or more CAPIs, or any combination of them, whose decreased abundance indicates the presence of an early inflammatory asthmatic response, *i.e* CAPI-1, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-20, CAPI-21, CAPI-24,

CAPI-25, CAPI-37 or CAPI-38, more preferably CAPI-1, CAPI-3, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-20, CAPI-24, CAPI-25, CAPI-37 or CAPI-38.

CAPIs modulated in the presence of a late inflammatory asthmatic response

In one embodiment, a bronchial biopsy is analyzed for quantitative detection of one or more of the following CAPIs: CAPI-1, CAPI-2, CAPI-3, CAPI-13, CAPI-15, CAPI-18, CAPI-21, CAPI-29, CAPI-30, CAPI-35 or CAPI-37 or any combination of them, wherein an increased abundance of the CAPI or CAPIs (or any combination of them) in the tissue from the subject relative to tissue from a subject or subjects free from chronic asthma (*e.g.*, a control sample or a previously determined reference range) indicates the presence of a late inflammatory asthmatic response. More preferably a bronchial biopsy (*e.g.*, from a subject suspected of having chronic asthma) is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CAPIs: CAPI-1, CAPI-15, CAPI-29 or CAPI-30, whose increased expression in the late inflammatory asthmatic response can be returned (at least partially) to basal levels by the administration of asthma-related therapies

In another embodiment, a bronchial biopsy is analyzed for quantitative detection of one or more of the following CAPIs: CAPI-5, CAPI-19, CAPI-25, CAPI-34 or CAPI-36 whose decreased expression indicates the presence of an early inflammatory asthmatic response. More preferably a bronchial biopsy (*e.g.*, from a subject suspected of having chronic asthma) is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CAPIs: CAPI-19, CAPI-25, CAPI-34 or CAPI-36 whose decreased expression in the late inflammatory asthmatic response can be returned (at least partially) to basal levels by the administration of asthma-related therapies

In a further embodiment, a bronchial biopsy is analyzed for quantitative detection of (a) one or more CAPIs, or any combination of them, whose increased abundance indicates the presence of a late inflammatory asthmatic response, *i.e.*, CAPI-1, CAPI-2, CAPI-3, CAPI-13, CAPI-15, CAPI-18, CAPI-21, CAPI-29, CAPI-30, CAPI-35 or CAPI-37, more preferably CAPI-1, CAPI-15, CAPI-29 or CAPI-30;

and (b) one or more CAPIs, or any combination of them, whose decreased abundance indicates the presence of an early inflammatory asthmatic response, *i.e* CAPI-5, CAPI-19, CAPI-25, CAPI-34 or CAPI-36, more preferably CAPI-19, CAPI-25, CAPI-34 or CAPI-36.

As shown above, the CAPIs described herein include previously unknown proteins, as well as variants of known proteins where the variants were not previously known to be associated with chronic asthma. For each CAPI, the present invention additionally provides: (a) a preparation comprising the isolated CAPI; (b) a preparation comprising one or more fragments of the CAPI; and (c) antibodies that bind to said CAPI, to said fragments, or both to said CAPI and to said fragments. As used herein, a CAPI is "isolated" when it is present in a preparation that is substantially free of contaminating proteins, *i.e.*, a preparation in which less than 10% (preferably less than 5%, more preferably less than 1%) of the total protein present is contaminating protein(s). A contaminating protein is a protein or protein isoform having a significantly different pI or MW from those of the isolated CAPI, as determined by 2D electrophoresis. As used herein, a "significantly different" pI or MW is one that permits the contaminating protein to be resolved from the CAPI on 2D electrophoresis, performed according to the Reference Protocol.

In one embodiment, an isolated protein is provided, said protein comprising a peptide with the amino acid sequence identified in Table IV for a CAPI, said protein having a pI and MW within 10% (preferably within 5%, more preferably within 1%) of the values identified in Table III for that CAPI.

The CAPIs of the invention can be qualitatively or quantitatively detected by any method known to those skilled in the art, including but not limited to the Preferred Technology described herein, kinase assays, enzyme assays, binding assays and other functional assays, immunoassays, and western blotting. In one embodiment, the CAPIs are separated on a 2-D gel by virtue of their MWs and pIs and visualized by staining the gel. In one embodiment, the CAPIs are stained with a fluorescent dye and imaged with a fluorescence scanner. Sypro Red (Molecular Probes, Inc., Eugene, Oregon) is a suitable dye for this purpose. A preferred fluorescent dye is Pyridinium,

4-[2-[4-(dipentylamino)-2-trifluoromethylphenyl] ethenyl]-1-(sulfobutyl)-, inner salt. See U.S. Application No. 09/412,168, filed on October 5, 1999, which is incorporated herein by reference in its entirety.

Alternatively, CAPIs can be detected in an immunoassay. In one embodiment, an immunoassay is performed by contacting a sample from a subject to be tested with an anti-CAPI antibody under conditions such that immunospecific binding can occur if the CAPI is present, and detecting or measuring the amount of any immunospecific binding by the antibody. Anti-CAPI antibodies can be produced by the methods and techniques taught herein; examples of such antibodies known in the art are set forth in Table V. These antibodies shown in Table V are already known to bind to the protein of which the CAPI is itself a family member. Preferably, the anti-CAPI antibody preferentially binds to the CAPI rather than to other variants of the same protein. In a preferred embodiment, the anti-CAPI antibody binds to the CAPI with at least 2-fold greater affinity, more preferably at least 5-fold greater affinity, still more preferably at least 10-fold greater affinity, than to said other variants of the same protein.

CAPIs can be transferred from the gel to a suitable membrane (*e.g.* a PVDF membrane) and subsequently probed in suitable assays that include, without limitation, competitive and non-competitive assay systems using techniques such as western blots and "sandwich" immunoassays using anti-CAPI antibodies as described herein, *e.g.*, the antibodies identified in Table VI, or others raised against the CAPIs of interest. The immunoblots can be used to identify those anti-CAPI antibodies displaying the selectivity required to immuno-specifically differentiate a CAPI from other isoforms encoded by the same gene.

Table V: Known Antibodies That Recognize CAPIs or CAPI-Related Polypeptides

Table V

| CAPI # | Antibody | Manufacturer | Catalogue # |
|---|---|---|---|
| CAPI-1 | Anti-Human Alpha Actinin, Mouse Monoclonal | Upstate Biotechnology | 05-384 |
| CAPI-2 | Anti-Human Alpha Actinin, Mouse Monoclonal | Upstate Biotechnology | 05-384 |
| CAPI-3 | Anti-Human Alpha Actinin, | Upstate Biotechnology | 05-384 |

Table V

| CAPI # | Antibody | M | Catalogue # |
|---|---|---|---|
| | Mouse Monoclonal | | |
| CAPI-5 | HSP 90 (H-114) | SANTA CRUZ BIOTECHNOLOGY, INC - RESEARCH ANTIBODIES 98/99 | sc-7947 |
| CAPI-13 | Anti-Cytokeratin Type 10 | RDI RESEARCH DIAGNOSTICS, INC | RDI-CBL196 |
| CAPI-18 | Rabbit anti-Annexin II monomer | BIODESIGN INTERNATIONAL | K80100R |
| CAPI-19 | Glyceraldehyde-3-Phosphate Dehydrogenase | BIODESIGN INTERNATIONAL | H86504M |

*Further information about these antibodies can be obtained from their commercial sources at: BIODESIGN INTERNATIONAL - http://www.biodesign.com/; RDI RESEARCH DIAGNOSTICS, INC - http://www.researchd.com/; Santa Cruz Biotechnology Inc – www.scbt.com/; Upstate Biotechnology www.upstatebiotech.com/.

In one embodiment, binding of antibody in tissue sections can be used to detect aberrant CAPI localization or an aberrant level of one or more CAPIs. In a specific embodiment, antibody to a CAPI can be used to assay a tissue sample (*e.g.*, a bronchial biopsy) from a subject for the level of the CAPI where an aberrant level of CAPI is indicative of chronic asthma. As used herein, an "aberrant level" means a level that is increased or decreased compared with the level in a subject free from chronic asthma or a reference level. If desired, the comparison can be performed with a matched sample from the same subject, taken from a portion of the body not affected by chronic asthma.

Any suitable immunoassay can be used, including, without limitation, competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays and protein A immunoassays.

For example, a CAPI can be detected in a fluid sample (*e.g.* blood, urine, or tissue homogenate) by means of a two-step sandwich assay.. In the first step, a capture reagent (*e.g.*, an anti-CAPI antibody) is used to capture the CAPI. Examples of such antibodies known in the art are set forth in Table V. The capture reagent can optionally be immobilized on a solid phase. In the second step, a directly or indirectly labeled detection reagent is used to detect the captured CAPI. In one embodiment, the detection reagent is a lectin. Any lectin can be used for this purpose that preferentially binds to the CAPI rather than to other isoforms that have the same core protein as the CAPI or to other proteins that share the antigenic determinant recognized by the antibody. In a preferred embodiment, the chosen lectin binds to the CAPI with at least 2-fold greater affinity, more preferably at least 5-fold greater affinity, still more preferably at least 10-fold greater affinity, than to said other isoforms that have the same core protein as the CAPI or to said other proteins that share the antigenic determinant recognized by the antibody. Based on the present description, a lectin that is suitable for detecting a given CAPI can readily be identified by methods well known in the art, for instance upon testing one or more lectins enumerated in Table I on pages 158-159 of Sumar et al., Lectins as Indicators of Disease-Associated Glycoforms, In: Gabius H-J & Gabius S (eds.), 1993, Lectins and Glycobiology, at pp. 158-174 (which is incorporated herein by reference in its entirety). Lectins with the desired oligosaccharide specificity can be identified, for example, by their ability to detect the CAPI in a 2D gel, in a replica of a 2D gel following transfer to a suitable solid substrate such as a nitrocellulose membrane, or in a two-step assay following capture by an antibody. In an alternative embodiment, the detection reagent is an antibody, *e.g.*, an antibody that immunospecifically detects other post-translational modifications, such as an antibody that immunospecifically binds to phosphorylated amino acids. Examples of such antibodies include those that bind to phosphotyrosine (BD Transduction Laboratories, catalog nos.: P11230-050/P11230-150; P11120; P38820; P39020), those that bind to phosphoserine (Zymed Laboratories Inc., South San Francisco, CA, catalog no. 61-8100) and those that bind to phosphothreonine (Zymed Laboratories Inc., South San Francisco, CA, catalog nos. 71-8200, 13-9200).

If desired, a gene encoding a CAPI, a related gene, or related nucleic acid sequences or subsequences, including complementary sequences, can also be used in hybridization assays. A nucleotide encoding a CAPI, or subsequences thereof comprising at least 8 nucleotides, preferably at least 12 nucleotides, and most preferably at least 15 nucleotides can be used as a hybridization probe. Hybridization assays can be used for detection, prognosis, diagnosis, or monitoring of conditions, disorders, or disease states, associated with aberrant expression of genes encoding CAPIs, or for differential diagnosis of subjects with signs or symptoms suggestive of chronic asthma. In particular, such a hybridization assay can be carried out by a method comprising contacting a subject's sample containing nucleic acid with a nucleic acid probe capable of hybridizing to a DNA or RNA that encodes a CAPI, under conditions such that hybridization can occur, and detecting or measuring any resulting hybridization. Nucleotides can be used for therapy of subjects having chronic asthma, as described below.

The invention also provides diagnostic kits, comprising an anti-CAPI antibody. In addition, such a kit may optionally comprise one or more of the following: (1) instructions for using the anti-CAPI antibody for diagnosis, prognosis, therapeutic monitoring or any combination of these applications; (2) a labeled binding partner to the antibody; (3) a solid phase (such as a reagent strip) upon which the anti-CAPI antibody is immobilized; and (4) a label or insert indicating regulatory approval for diagnostic, prognostic or therapeutic use or any combination thereof. If no labeled binding partner to the antibody is provided, the anti-CAPI antibody itself can be labeled with a detectable marker, *e.g.*, a chemiluminescent, enzymatic, fluorescent, or radioactive moiety.

The invention also provides a kit comprising a nucleic acid probe capable of hybridizing to RNA encoding a CAPI. In a specific embodiment, a kit comprises in one or more containers a pair of primers (*e.g.*, each in the size range of 6-30 nucleotides, more preferably 10-30 nucleotides and still more preferably 10-20 nucleotides) that under appropriate reaction conditions can prime amplification of at least a portion of a nucleic acid encoding a CAPI, such as by polymerase chain

reaction (see, *e.g.*, Innis et al., 1990, PCR Protocols, Academic Press, Inc., San Diego, CA), ligase chain reaction (see EP 320,308) use of Qβ replicase, cyclic probe reaction, or other methods known in the art.

Kits are also provided which allow for the detection of a plurality of CAPIs or a plurality of nucleic acids each encoding a CAPI. A kit can optionally further comprise a predetermined amount of an isolated CAPI protein or a nucleic acid encoding a CAPI, *e.g.*, for use as a standard or control.

## 5.4 Statistical Techniques for Identifying CAPIs and CAPI Clusters

The uni-variate differential analysis tools, such as fold changes, Wilcoxon rank sum test and t-test, are useful in identifying individual CAFs or CAPIs that are diagnostically associated with chronic asthma or in identifying individual CAPIs that regulate the disease process. In most cases, however, those skilled in the art appreciate that the disease process is associated with a combination of CAFs or CAPIs (and to be regulated by a combination of CAPIs), rather than individual CAFs and CAPIs in isolation. The strategies for discovering such combinations of CAFs and CAPIs differ from those for discovering individual CAFs and CAPIs. In such cases, each individual CAF and CAPI can be regarded as one variable and the disease can be regarded as a joint, multi-variate effect caused by interaction of these variables.

The following steps can be used to identify markers from data produced by the Preferred Technology.

The first step is to identify a collection of CAFs or CAPIs that individually show significant association with chronic asthma. The association between the identified CAFs or CAPIs and chronic asthma need not be as highly significant as is desirable when an individual CAF or CAPI is used as a diagnostic. Any of the tests discussed above (fold changes, Wilcoxon rank sum test, etc.) can be used at this stage. Once a suitable collection of CAFs or CAPIs has been identified, a sophisticated multi-variate analysis capable of identifying clusters can then be used to estimate the significant multivariate associations with chronic asthma.

Linear Discriminant Analysis (LDA) is one such procedure, which can be used to detect significant association between a cluster of variables (*i.e.*, CAFs or CAPIs) and chronic asthma. In performing LDA, a set of weights is associated with each variable (*i.e.*, CAF or CAPI) so that the linear combination of weights and the measured values of the variables can identify the disease state by discriminating between subjects having chronic asthma and subjects free from chronic asthma. Enhancements to the LDA allow stepwise inclusion (or removal) of variables to optimize the discriminant power of the model. The result of the LDA is therefore a cluster of CAFs or CAPIs which can be used, without limitation, for diagnosis, prognosis, therapy or drug development. Other enhanced variations of LDA, such as Flexible Discriminant Analysis permit the use of non-linear combinations of variables to discriminate a disease state from a normal state. The results of the discriminant analysis can be verified by post-hoc tests and also by repeating the analysis using alternative techniques such as classification trees.

A further category of CAFs or CAPIs can be identified by qualitative measures by comparing the percentage feature presence of a CAF or CAPI of one group of samples (*e.g.*, samples from diseased subjects) with the percentage feature presence of a CAF or CAPI in another group of samples (*e.g.*, samples from control subjects). The "percentage feature presence" of a CAF or CAPI is the percentage of samples in a group of samples in which the CAF or CAPI is detectable by the detection method of choice. For example, if a CAF is detectable in 95 percent of samples from diseased subjects, the percentage feature presence of that CAF in that sample group is 95 percent. If only 5 percent of samples from non-diseased subjects have detectable levels of the same CAF, detection of that CAF in the sample of a subject would suggest that it is likely that the subject suffers from chronic asthma.

## 5.5 Use in Clinical Studies

The diagnostic methods and compositions of the present invention can assist in monitoring a clinical study, *e.g.* to evaluate drugs for therapy of chronic asthma. In one embodiment, candidate molecules are tested for their ability to restore CAF or

CAPI levels in a subject having chronic asthma to levels found in subjects free from chronic asthma or, in a treated subject (*e.g.* after treatment with a cholinesterase inhibitor), to preserve CAF or CAPI levels at or near non-asthmatic values. The levels of one or more CAFs or CAPIs can be assayed.

In another embodiment, the methods and compositions of the present invention are used to screen candidates for a clinical study to identify individuals having chronic asthma; such individuals can then be excluded from the study or can be placed in a separate cohort for treatment or analysis.

In another embodiment, the methods and compositions of the present invention are used to screen for individuals most likely to respond to treatment with a given chronic asthma therapeutic agent (e.g. patients displaying a chronic asthma antigen for which a specific antibody therapy has been developed.

## 5.6 Purification of CAPIs

In particular aspects, the invention provides isolated mammalia CAPIs, preferably huma CAPIs, and fragments thereof which comprise an antigenic determinant (*i.e.*, can be recognized by an antibody) or which are otherwise functionally active, as well as nucleic acid sequences encoding the foregoing. "Functionally active"as used herein refers to material displaying one or more functional activities associated with a full-length (wild-type) CAPI, *e.g.*, binding to a CAPI substrate or CAPI binding partner, antigenicity (binding to an anti-CAPI antibody), immunogenicity, enzymatic activity and the like.

In specific embodiments, the invention provides fragments of a CAPI comprising at least 5 amino acids, at least 10 amino acids, at least 50 amino acids, or at least 75 amino acids. Fragments lacking some or all of the regions of a CAPI are also provided, as are proteins (*e.g.*, fusion proteins) comprising such fragments. Nucleic acids encoding the foregoing are provided.

Once a recombinant nucleic acid which encodes the CAPI, a portion of the CAPI, or a precursor of the CAPI is identified, the gene product can be analyzed. This is achieved by assays based on the physical or functional properties of the product,

including radioactive labeling of the product followed by analysis by gel electrophoresis, immunoassay, etc.

The CAPIs identified herein can be isolated and purified by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

Alternatively, once a recombinant nucleic acid that encodes the CAPI is identified, the entire amino acid sequence of the CAPI can be deduced from the nucleotide sequence of the gene coding region contained in the recombinant nucleic acid. As a result, the protein can be synthesized by standard chemical methods known in the art (*e.g.*, see Hunkapiller et al., 1984, Nature 310:105-111).

In another alternative embodiment, native CAPIs can be purified from natural sources, by standard methods such as those described above (*e.g.*, immunoaffinity purification).

In a preferred embodiment, CAPIs are isolated by the Preferred Technology described supra. For preparative-scale runs, a narrow-range "zoom gel" having a pH range of 2 pH units or less is preferred for the isoelectric step, according to the method described in Westermeier, 1993, Electrophoresis in Practice (VCH, Weinheim, Germany), pp. 197-209 (which is incorporated herein by reference in its entirety); this modification permits a larger quantity of a target protein to be loaded onto the gel, and thereby increases the quantity of isolated CAPI that can be recovered from the gel. When used in this way for preparative-scale runs, the Preferred Technology typically provides up to 100 ng, and can provide up to 1000 ng, of an isolated CAPI in a single run. Those of skill in the art will appreciate that a zoom gel can be used in any separation strategy which employs gel isoelectric focusing.

The invention thus provides an isolated CAPI, an isolated CAPI-related polypeptide, and an isolated derivative or fragment of a CAPI or a CAPI-related polypeptide; any of the foregoing can be produced by recombinant DNA techniques or by chemical synthetic methods.

## 5.7 Isolation of DNA Encoding a CAPI

Particular embodiments for the cloning of a gene encoding a CAPI, are presented below by way of example and not of limitation.

The nucleotide sequences of the present invention, including DNA and RNA, and comprising a sequence encoding a CAPI or a fragment thereof, or a CAPI-related polypeptide, may be synthesized using methods known in the art, such as using conventional chemical approaches or polymerase chain reaction (PCR) amplification. The nucleotide sequences of the present invention also permit the identification and cloning of the gene encoding a CAPI homolog or CAPI ortholog including, for example, by screening cDNA libraries, genomic libraries or expression libraries.

For example, to clone a gene encoding a CAPI by PCR techniques, anchored degenerate oligonucleotides (or a set of most likely oligonucleotides) can be designed for all CAPI peptide fragments identified as part of the same protein. PCR reactions under a variety of conditions can be performed with relevant cDNA and genomic DNAs (*e.g.*, from upper airways tissue or from cells of the immune system) from one or more species. Also vectorette reactions can be performed on any available cDNA and genomic DNA using the oligonucleotides (which preferably are nested) as above. Vectorette PCR is a method that enables the amplification of specific DNA fragments in situations where the sequence of only one primer is known. Thus, it extends the application of PCR to stretches of DNA where the sequence information is only available at one end. (Arnold C, 1991, PCR Methods Appl. 1(1):39-42; Dyer KD, Biotechniques, 1995, 19(4):550-2). Vectorette PCR may pe performed with probes that are, for example, anchored degenerate oligonucleotides (or most likely oligonucleotides) coding for CAPI peptide fragments, using as a template a genomic library or cDNA library pools.

Anchored degenerate oligonucleotides (and most likely oligonucleotides) can be designed for all CAPI peptide fragments. These oligonucleotides may be labelled and hybridized to filters containing cDNA and genomic DNA libraries. Oligonucleotides to different peptides from the same protein will often identify the

same members of the library. The cDNA and genomic DNA libraries may be obtained from any suitable or desired mammalian species, for example from humans.

Nucleotide sequences comprising a nucleotide sequence encoding a CAPI or CAPI fragment of the present invention are useful for their ability to hybridize selectively with complementary stretches of genes encoding other proteins. Depending on the application, a variety of hybridization conditions may be employed to obtain nucleotide sequences at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99% identical, or 100% identical, to the sequence of a nucleotide encoding a CAPI.

For a high degree of selectivity, relatively stringent conditions are used to form the duplexes, such as low salt or high temperature conditions. As used herein, "highly stringent conditions" means hybridization to filter-bound DNA in 0.5 M NaHPO4, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65□C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3; incorporated herein by reference in its entirety.) For some applications, less stringent conditions for duplex formation are required. As used herein "moderately stringent conditions" means washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, supra). Hybridization conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilize the hybrid duplex. Thus, particular hybridization conditions can be readily manipulated, and will generally be chosen depending on the desired results. In general, convenient hybridization temperatures in the presence of 50% formamide are: 42°C for a probe which is 95 to 100% identical to the fragment of a gene encoding a CAPI, 37°C for 90 to 95% identity and 32°C for 70 to 90% identity.

In the preparation of genomic libraries, DNA fragments are generated, some of which will encode parts or the whole of a CAPI. Any suitable method for preparing DNA fragments may be used in the present invention. For example, the DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use

DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The DNA fragments can then be separated according to size by standard techniques, including but not limited to agarose and polyacrylamide gel electrophoresis, column chromatography and sucrose gradient centrifugation. The DNA fragments can then be inserted into suitable vectors, including but not limited to plasmids, cosmids, bacteriophages lambda or T4, and yeast artificial chromosome (YAC). (See, *e.g.*, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II; Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York). The genomic library may be screened by nucleic acid hybridization to labeled probe (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961).

Based on the present description, the genomic libraries may be screened with labeled degenerate oligonucleotide probes corresponding to the amino acid sequence of any peptide of the CAPI using optimal approaches well known in the art. Any probe used is at least 10 nucleotides, at least 15 nucleotides, at least 20 nucleotides, at least 25 nucleotides, at least 30 nucleotides, at least 40 nucleotides, at least 50 nucleotides, at least 60 nucleotides, at least 70 nucleotides, at least 80 nucleotides, or at least 100 nucleotides. Preferably a probe is 10 nucleotides or longer, and more preferably 15 nucleotides or longer.

In Table IV above, CAPIs disclosed herein were found to correspond to variants of previously identified proteins encoded by genes whose sequences are publicly known. (Sequence analysis and protein identification of CAPIs was carried out using the methods described in Section 6.1.14). To screen for any of the above mentioned genes, any probe may be used that is complementary to the gene or its complement; preferably the probe is 10 nucleotides or longer, more preferably 15 nucleotides or longer. The SWISS-PROT and trEMBL databases (held by the Swiss Institute of Bioinformatics (SIB) and the European Bioinformatics Institue (EBI)

which are available at http://www.expasy.ch/) and the GenBank database (held by the National Institute of Health (NIH) which is available at http://www.ncbi.nlm.nih.gov/GenBank/) provide sequences for the CAPIs listed in Table IV under the following accession numbers and each sequence is incorporated herein by reference.

Table VI: Nucleotide Sequences Encoding CAPIs or CAPI-related proteins.

Table VI

| CAF # | CAPI # | Accession Numbers |
|---|---|---|
| CAF-1 | CAPI-1 | 4826639 |
| CAF-2 | CAPI-2 | X15804.1 |
| CAF-3 | CAPI-3 | O43707 |
| CAF-5 | CAPI-5 | P08238 |
| CAF-12 | CAPI-12 | P36871 |
| CAF-29 | CAPI-29 | 12803081 |
| CAF-29 | CAPI-30 | P54577 |
| CAF-13 | CAPI-13 | P13645 |
| CAF-13 | CAPI-37 | AF077226.2 |
| CAF-15 | CAPI-15 | Q16401 |
| CAF-18 | CAPI-18 | P07355 |
| CAF-19 | CAPI-19 | P04406 |
| CAF-34 | CAPI-35 | P48739 |
| CAF-20 | CAPI-38 | 2895085 |
| CAF-20 | CAPI-20 | 4507643 |
| CAF-21 | CAPI-21 | Q15056 |
| CAF-22 | CAPI-22 | D10656.1 |
| CAF-24 | CAPI-24 | X04098.1 |
| CAF-25 | CAPI-25 | P05388 |
| CAF-33 | CAPI-34 | P14625 |
| CAF-35 | CAPI-36 | P30086 |

For any CAPI, degenerate probes, or probes taken from the sequences described above by accession number may be used for screening. In the case of degenerate probes, they can be constructed from the partial amino sequence information obtained from tandem mass spectra of tryptic digest peptides of the CAPI. To screen such a gene, any probe may be used that is complementary to the gene or its

complement; preferably the probe is 10 nucleotides or longer, more preferably 15 nucleotides or longer. When a library is screened, clones with insert DNA encoding the CAPI or a fragment thereof will hybridize to one or more members of the corresponding set of degenerate oligonucleotide probes (or their complement). Hybridization of such oligonucleotide probes to genomic libraries is carried out using methods known in the art. For example, hybridization with one of the above-mentioned degenerate sets of oligonucleotide probes, or their complement (or with any member of such a set, or its complement) can be performed under highly stringent or moderately stringent conditions as defined above, or can be carried out in 2X SSC, 1.0% SDS at 50°C and washed using the washing conditions described supra for highly stringent or moderately stringent hybridization.

In yet another aspect of the invention, clones containing nucleotide sequences encoding the entire CAPI, a fragment of a CAPI, a CAPI-related polypeptide, or a fragment of a CAPI-related polypeptide any of the foregoing may also be obtained by screening expression libraries. For example, DNA from the relevant source is isolated and random fragments are prepared and ligated into an expression vector (*e.g.*, a bacteriophage, plasmid, phagemid or cosmid) such that the inserted sequence in the vector is capable of being expressed by the host cell into which the vector is then introduced. Various screening assays can then be used to select for the expressed CAPI or CAPI-related polypeptides. In one embodiment, the various anti-CAPI antibodies of the invention can be used to identify the desired clones using methods known in the art. See, for example, Harlow and Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Appendix IV. Colonies or plaques from the library are brought into contact with the antibodies to identify those clones that bind antibody.

In an embodiment, colonies or plaques containing DNA that encodes a CAPI, a fragment of a CAPI, a CAPI-related polypeptide, or a fragment of a CAPI-related polypeptide can be detected using DYNA Beads according to Olsvick et al., 29th ICAAC, Houston, Tex. 1989, incorporated herein by reference. Anti-CAPI antibodies

are crosslinked to tosylated DYNA Beads M280, and these antibody-containing beads are then contacted with colonies or plaques expressing recombinant polypeptides. Colonies or plaques expressing a CAPI or CAPI-related polypeptide are identified as any of those that bind the beads.

Alternatively, the anti-CAPI antibodies can be nonspecifically immobilized to a suitable support, such as silica or Celite7 resin. This material is then used to adsorb to bacterial colonies expressing the CAPI protein or CAPI-related polypeptide as described herein.

In another aspect, PCR amplification may be used to isolate from genomic DNA a substantially pure DNA (*i.e.*, a DNA substantially free of contaminating nucleic acids) encoding the entire CAPI or a part thereof. Preferably such a DNA is at least 95% pure, more preferably at least 99% pure. Oligonucleotide sequences, degenerate or otherwise, that correspond to peptide sequences of CAPIs disclosed herein can be used as primers.

PCR can be carried out, *e.g.*; by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp7 or AmpliTaq DNA polymerase). One can choose to synthesize several different degenerate primers, for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the degenerate primers and the corresponding sequences in the DNA. After successful amplification of a segment of the sequence encoding a CAPI, that segment may be molecularly cloned and sequenced, and utilized as a probe to isolate a complete genomic clone. This, in turn, will permit the determination of the gene's complete nucleotide sequence, the analysis of its expression, and the production of its protein product for functional analysis, as described *infra*.

The gene encoding a CAPI can also be identified by mRNA selection by nucleic acid hybridization followed by in vitro translation. In this procedure, fragments are used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified DNA encoding a CAPI of another species (*e.g.*, mouse, human). Immunoprecipitation analysis or functional assays (*e.g.*,

aggregation ability in vitro; binding to receptor) of the in vitro translation products of the isolated products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that contain the desired sequences. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies that specifically recognize a CAPI. A radiolabelled cDNA encoding a CAPI can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabelled mRNA or cDNA may then be used as a probe to identify the DNA fragments encoding a CAPI from among other genomic DNA fragments.

Alternatives to isolating genomic DNA encoding a CAPI include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the CAPI. For example, RNA for cDNA cloning of the gene encoding a CAPI can be isolated from cells which express the CAPI. Those skilled in the art will understand from the present description that other methods may be used and are within the scope of the invention.

Any suitable eukaryotic cell can serve as the nucleic acid source for the molecular cloning of the gene encoding a CAPI. The nucleic acid sequences encoding the CAPI can be isolated from vertebrate, mammalian, primate, human, porcine, bovine, feline, avian, equine, canine or murine sources. The DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell. (See, *e.g.*, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences.

The identified and isolated gene or cDNA can then be inserted into any suitable cloning vector. A large number of vector-host systems known in the art may be used. As those skilled in the art will appreciate, the only limitation is that the

vector system chosen be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, plasmids such as PBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene) or modified viruses such as adenoviruses, adeno-associated viruses or retroviruses. The insertion into a cloning vector can be accomplished, for example, by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and the gene encoding a CAPI may be modified by homopolymeric tailing. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the isolated gene encoding the CAPI, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The nucleotide sequences of the present invention include nucleotide sequences encoding amino acid sequences with substantially the same amino acid sequences as native CAPIs, nucleotide sequences encoding amino acid sequences with functionally equivalent amino acids, nucleotide sequences encoding CAPIs, a fragment of CAPIs, CAPI-related polypeptides, or fragments of CAPI-related polypeptides.

In a specific embodiment, an isolated nucleic acid molecule encoding a CAPI-related polypeptide can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of a CAPI such that

one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Standard techniques known to those of skill in the art can be used to introduce mutations, including, for example, site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a side chain with a similar charge. Families of amino acid residues having side chains with similar charges have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Alternatively, mutations can be introduced randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed and the activity of the protein can be determined.

## 5.8 Expression of DNA Encoding CAPIs

The nucleotide sequence coding for a CAPI, a CAPI analog, a CAPI-related peptide, or a fragment or other derivative of any of the foregoing, can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native gene encoding the CAPI or its flanking regions, or the native gene encoding the CAPI-related polypeptide or its flanking regions. A variety of host-vector systems may be utilized in the present invention to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus);

microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. In specific embodiments, a nucleotide sequence encoding a human gene (or a nucleotide sequence encoding a functionally active portion of a huma CAPI) is expressed. In yet another embodiment, a fragment of a CAPI comprising a domain of the CAPI is expressed.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional and translational control signals and the protein coding sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinants (genetic recombination). Expression of nucleic acid sequence encoding a CAPI or fragment thereof may be regulated by a second nucleic acid sequence so that the CAPI or fragment is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a CAPI may be controlled by any promoter or enhancer element known in the art. Promoters which may be used to control the expression of the gene encoding a CAPI or a CAPI-related polypeptide include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), the tetracycline (Tet) promoter (Gossen et al., 1995, Proc. Nat. Acad. Sci. USA 89:5547-5551); prokaryotic expression vectors such as the _-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25; see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94); plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or

the cauliflower mosaic virus 35S RNA promoter (Gardner, et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286); neuronal-specific enolase (NSE) which is active in neuronal cells (Morelli et al., 1999, Gen. Virol. 80:571-83).

In a specific embodiment, a vector is used that comprises a promoter operably linked to a CAPI-encoding nucleic acid, one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene).

In a specific embodiment, an expression construct is made by subcloning a CAPI or a CAPI-related polypeptide coding sequence into the EcoRI restriction site of each of the three pGEX vectors (Glutathione S-Transferase expression vectors; Smith and Johnson, 1988, Gene 7:31-40). This allows for the expression of the CAPI product or CAPI-related polypeptide from the subclone in the correct reading frame.

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the CAPI coding sequence or CAPI-related polypeptide coding sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the antibody molecule in infected hosts (*e.g.*, see Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:355-359). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:51-544).

Expression vectors containing inserts of a gene encoding a CAPI or a CAPI-related polypeptide can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a gene encoding a CAPI inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted gene encoding a CAPI. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions

(*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of a gene encoding a CAPI in the vector. For example, if the gene encoding the CAPI is inserted within the marker gene sequence of the vector, recombinants containing the gene encoding the CAPI insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the gene product (*i.e.*, CAPI) expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the CAPI in in vitro assay systems, *e.g.*, binding with anti-CAPI antibody.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered CAPI or CAPI-related polypeptide may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, phosphorylation of proteins). Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system will produce an unglycosylated product and expression in yeast will produce a glycosylated product. Eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERY, BHK, Hela, COS, MDCK, HEK293, 3T3, WI38. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the differentially expressed or pathway gene protein may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable

marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched medium, and then are switched to a selective medium. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the differentially expressed or pathway gene protein. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the differentially expressed or pathway gene protein.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes.

In other specific embodiments, the CAPI, fragment, analog, or derivative may be expressed as a fusion, or chimeric protein product (comprising the protein, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence). For example, the polypeptides of the present invention may be fused with the constant domain of immunoglobulins (IgA, IgE, IgG, IgM), or portions thereof (CH1, CH2, CH3, or any combination thereof and portions thereof) resulting in chimeric polypeptides. Such fusion proteins may facilitate purification, increase half-life in vivo, and enhance the delivery of an antigen across an epithelial barrier to the immune system. An increase in the half-life in vivo and facilitated purification has

been shown for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. See, *e.g.*, EP 394,827; Traunecker et al., Nature, 331:84-86 (1988). Enhanced delivery of an antigen across the epithelial barrier to the immune system has been demonstrated for antigens (*e.g.*, insulin) conjugated to an FcRn binding partner such as IgG or Fc fragments (see, *e.g.*, PCT publications WO 96/22024 and WO 99/04813).

Nucleic acids encoding a CAPI, a fragment of a CAPI, a CAPI-related polypeptide, or a fragment of a CAPI-related polypeptide can fused to an epitope tag (*e.g.*, the hemagglutinin ("HA") tag or flag tag) to aid in detection and purification of the expressed polypeptide. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht et al., 1991, Proc. Natl. Acad. Sci. USA 88:8972-897).

Fusion proteins can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, a fusion protein may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer.

Both cDNA and genomic sequences can be cloned and expressed.

## 5.9 Domain Structure of CAPIs

Domains of some CAPIs are known in the art and have been described in the scientific literature. Moreover, domains of a CAPI can be identified using techniques known to those of skill in the art. For example, one or more domains of a CAPI can be identified by using one or more of the following programs: ProDom, TMpred, and SAPS. ProDom compares the amino acid sequence of a polypeptide to a database of compiled domains (see, *e.g.*, http://www.toulouse.inra.fr/prodom.html; Corpet F., Gouzy J. & Kahn D., 1999, Nucleic Acids Res., 27:263-267). TMpred predicts membrane-spanning regions of a polypeptide and their orientation. This program uses an algorithm that is based on the statistical analysis of TMbase, a database of naturally

occuring transmembrane proteins (see, *e.g.*, http://www.ch.embnet.org/software/TMPRED_form.html; Hofmann & Stoffel. (1993) ATMbase - A database of membrane spanning proteins segments." Biol. Chem. Hoppe-Seyler 347,166). The SAPS program analyzes polypeptides for statistically significant features like charge-clusters, repeats, hydrophobic regions, compositional domains (see, *e.g.*, Brendel et al., 1992, Proc. Natl. Acad. Sci. USA 89: 2002-2006). Thus, based on the present description, the skilled artisan can identify domains of a CAPI having enzymatic or binding activity, and further can identify nucleotide sequences encoding such domains. These nucleotide sequences can then be used for recombinant expression of a CAPI fragment that retains the enzymatic or binding activity of the CAPI.

Based on the present description, the skilled artisan can identify domains of a CAPI having enzymatic or binding activity, and further can identify nucleotide sequences encoding such domains. These nucleotide sequences can then be used for recombinant expression of CAPI fragments that retain the enzymatic or binding activity of the CAPI.

In one embodiment, a CAPI has an amino acid sequence sufficiently similar to an identified domain of a known polypeptide. As used herein, the term "sufficiently similar" refers to a first amino acid or nucleotide sequence which contains a sufficient number of identical or equivalent (*e.g.*, with a similar side chain) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have or encode a common structural domain or common functional activity or both.

A CAPI domain can be assessed for its function using techniques well known to those of skill in the art. For example, a domain can be assessed for its kinase activity or for its ability to bind to DNA using techniques known to the skilled artisan. Kinase activity can be assessed, for example, by measuring the ability of a polypeptide to phosphorylate a substrate. DNA binding activity can be assessed, for example, by measuring the ability of a polypeptide to bind to a DNA binding element in a electromobility shift assay.

## 5.10 Producti n of Antibodies to CAPIs

According to the invention a CAPI, CAPI analog, CAPI-related protein or a fragment or derivative of any of the foregoing may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such immunogens can be isolated by any convenient means, including the methods described above. Antibodies of the invention include, but are not limited to polyclonal, monoclonal, bispecific, humanized or chimeric antibodies; single chain antibodies, Fab fragments and F(ab') fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen-binding site that specifically binds an antigen. The immunoglobulin molecules of the invention can be of any class (*e.g.*, IgG, IgE, IgM, IgD and IgA ) or subclass of immunoglobulin molecule.

In one embodiment, antibodies that recognize gene products of genes encoding CAPIs are publicly available. For example, antibodies that recognize these CAPIs and/or their isoforms include antibodies recognizing, CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-21, CAPI-22, CAPI-24 and CAPI-25 and antibodies to some of which can be purchased from commercial sources as shown in Table V above. In another embodiment, methods known to those skilled in the art are used to produce antibodies that recognize a CAPI, a CAPI analog, a CAPI-related polypeptide, or a derivative or fragment of any of the foregoing.

In one embodiment of the invention, antibodies to a specific domain of a CAPI are produced. In a specific embodiment, hydrophilic fragments of a CAPI are used as immunogens for antibody production.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.* ELISA (enzyme-linked

immunosorbent assay). For example, to select antibodies which recognize a specific domain of a CAPI, one may assay generated hybridomas for a product which binds to a CAPI fragment containing such domain. For selection of an antibody that specifically binds a first CAPI homolog but which does not specifically bind to (or binds less avidly to) a second CAPI homolog, one can select on the basis of positive binding to the first CAPI homolog and a lack of binding to (or reduced binding to) the second CAPI homolog. Similarly, for selection of an antibody that specifically binds a CAPI but which does not specifically bind to (or binds less avidly to) a different isoform of the same protein (such as a different glycoform having the same core peptide as the CAPI), one can select on the basis of positive binding to the CAPI and a lack of binding to (or reduced binding to) the different isoform (*e.g.*, a different glycoform). Thus, the present invention provides an antibody (preferably a monoclonal antibody) that binds with greater affinity (preferably at least 2-fold, more preferably at least 5-fold still more preferably at least 10-fold greater affinity) to a CAPI than to a different isoform or isoforms (*e.g.*, glycoforms) of the CAPI.

Polyclonal antibodies which may be used in the methods of the invention are heterogeneous populations of antibody molecules derived from the sera of immunized animals. Unfractionated immune serum can also be used. Various procedures known in the art may be used for the production of polyclonal antibodies to a CAPI, a fragment of a CAPI, a CAPI-related polypeptide, or a fragment of a CAPI-related polypeptide. In a particular embodiment, rabbit polyclonal antibodies to an epitope of a CAPI or a CAPI-related polypeptide can be obtained. For example, for the production of polyclonal or monoclonal antibodies, various host animals can be immunized by injection with the native or a synthetic (*e.g.*, recombinant) version of a CAPI, a fragment of a CAPI, a CAPI-related polypeptide, or a fragment of a CAPI-related polypeptide, including but not limited to rabbits, mice, rats, etc. The Preferred Technology described herein provides isolated CAPIs suitable for such immunization. If the CAPI is purified by gel electrophoresis, the CAPI can be used for immunization with or without prior extraction from the polyacrylamide gel. Various adjuvants may be used to enhance the immunological response, depending on the host species,

including, but not limited to, complete or incomplete Freund's adjuvant, a mineral gel such as aluminum hydroxide, surface active substance such as lysolecithin, pluronic polyol, a polyanion, a peptide, an oil emulsion, keyhole limpet hemocyanin, dinitrophenol, and an adjuvant such as BCG (bacille Calmette-Guerin) or corynebacterium parvum. Additional adjuvants are also well known in the art.

For preparation of monoclonal antibodies (mAbs) directed toward a CAPI, a fragment of a CAPI, a CAPI-related polypeptide, or a fragment of a CAPI-related polypeptide, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAbs of the invention may be cultivated in vitro or in vivo. In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing known technology (PCT/US90/02545, incorporated herein by reference).

The monoclonal antibodies include but are not limited to human monoclonal antibodies and chimeric monoclonal antibodies (*e.g.*, human-mouse chimeras). A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a human immunoglobulin constant region and a variable region derived from a murine mAb. (See, *e.g.*, Cabilly et al., U.S. Patent No. 4,816,567; and Boss et al., U.S. Patent No. 4,816397, which are incorporated herein by reference in their entirety.) Humanized antibodies are antibody molecules from non-human species having one or more complementarily determining regions (CDRs) from the non-human species and a framework region from a human immunoglobulin molecule. (See, *e.g.*, Queen, U.S. Patent No. 5,585,089, which is incorporated herein by reference in its entirety.)

Chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in PCT Publication No. WO 87/02671; European Patent Application 184,187; European Patent Application 171,496; European Patent Application 173,494; PCT Publication No. WO 86/01533; U.S. Patent No. 4,816,567; European Patent Application 125,023; Better et al., 1988, Science 240:1041-1043; Liu et al., 1987, Proc. Natl. Acad. Sci. USA 84:3439-3443; Liu et al., 1987, J. Immunol. 139:3521-3526; Sun et al., 1987, Proc. Natl. Acad. Sci. USA 84:214-218; Nishimura et al., 1987, Canc. Res. 47:999-1005; Wood et al., 1985, Nature 314:446-449; and Shaw et al., 1988, J. Natl. Cancer Inst. 80:1553-1559; Morrison, 1985, Science 229:1202-1207; Oi et al., 1986, Bio/Techniques 4:214; U.S. Patent 5,225,539; Jones et al., 1986, Nature 321:552-525; Verhoeyan et al. (1988) Science 239:1534; and Beidler et al., 1988, J. Immunol. 141:4053-4060.

Completely human antibodies are particularly desirable for therapeutic treatment of human subjects. Such antibodies can be produced using transgenic mice which are incapable of expressing endogenous immunoglobulin heavy and light chains genes, but which can express human heavy and light chain genes. The transgenic mice are immunized in the normal fashion with a selected antigen, *e.g.*, all or a portion of a CAPI of the invention. Monoclonal antibodies directed against the antigen can be obtained using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar (1995, Int. Rev. Immunol. 13:65-93). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, *e.g.*, U.S. Patent 5,625,126; U.S. Patent 5,633,425; U.S. Patent 5,569,825; U.S. Patent 5,661,016; and U.S. Patent 5,545,806. In addition, companies such as Abgenix, Inc. (Freemont, CA) and

Genpharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

Completely human antibodies that recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, *e.g.*, a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope (Jespers et al. (1994) Bio/technology 12:899-903).

The antibodies of the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In a particular, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library (*e.g.*, human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, *e.g.*, using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman et al., J. Immunol. Methods 182:41-50 (1995); Ames et al., J. Immunol. Methods 184:177-186 (1995); Kettleborough et al., Eur. J. Immunol. 24:952-958 (1994); Persic et al., Gene 187 9-18 (1997); Burton et al., Advances in Immunology 57:191-280 (1994); PCT Application No. PCT/GB91/01134; PCT Publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies,

including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, *e.g.*, as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and F(ab')2 fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax et al., BioTechniques 12(6):864-869 (1992); and Sawai et al., AJRI 34:26-34 (1995); and Better et al., Science 240:1041-1043 (1988) (said references incorporated by reference in their entireties).

Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston et al., Methods in Enzymology 203:46-88 (1991); Shu et al., PNAS 90:7995-7999 (1993); and Skerra et al., Science 240:1038-1040 (1988).

The invention further provides for the use of bispecific antibodies, which can be made by methods known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Milstein et al., 1983, Nature 305:537-539). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., 1991, EMBO J. 10:3655-3659.

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired,

the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published March 3,1994. For further details for generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 1986, 121:210.

The invention provides functionally active fragments, derivatives or analogs of the anti-CAPI immunoglobulin molecules. Functionally active means that the fragment, derivative or analog is able to elicit anti-anti-idiotype antibodies (*i.e.*, tertiary antibodies) that recognize the same antigen that is recognized by the antibody from which the fragment, derivative or analog is derived. Specifically, in a preferred embodiment the antigenicity of the idiotype of the immunoglobulin molecule may be enhanced by deletion of framework and CDR sequences that are C-terminal to the CDR sequence that specifically recognizes the antigen. To determine which CDR sequences bind the antigen, synthetic peptides containing the CDR sequences can be used in binding assays with the antigen by any binding assay method known in the art.

The present invention provides antibody fragments such as, but not limited to, F(ab')2 fragments and Fab fragments. Antibody fragments which recognize specific

epitopes may be generated by known techniques. F(ab')2 fragments consist of the variable region, the light chain constant region and the CH1 domain of the heavy chain and are generated by pepsin digestion of the antibody molecule. Fab fragments are generated by reducing the disulfide bridges of the F(ab')2 fragments. The invention also provides heavy chain and light chain dimers of the antibodies of the invention, or any minimal fragment thereof such as Fvs or single chain antibodies (SCAs) (*e.g.*, as described in U.S. Patent 4,946,778; Bird, 1988, Science 242:423-42; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-54), or any other molecule with the same specificity as the antibody of the invention. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Techniques for the assembly of functional Fv fragments in E. coli may be used (Skerra et al., 1988, Science 242:1038-1041).

In other embodiments, the invention provides fusion proteins of the immunoglobulins of the invention (or functionally active fragments thereof), for example in which the immunoglobulin is fused via a covalent bond (*e.g.*, a peptide bond), at either the N-terminus or the C-terminus to an amino acid sequence of another protein (or portion thereof, preferably at least 10, 20 or 50 amino acid portion of the protein) that is not the immunoglobulin. Preferably the immunoglobulin, or fragment thereof, is covalently linked to the other protein at the N-terminus of the constant domain. As stated above, such fusion proteins may facilitate purification, increase half-life in vivo, and enhance the delivery of an antigen across an epithelial barrier to the immune system.

The immunoglobulins of the invention include analogs and derivatives that are either modified, i.e, by the covalent attachment of any type of molecule as long as such covalent attachment that does not impair immunospecific binding. For example, but not by way of limitation, the derivatives and analogs of the immunoglobulins include those that have been further modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphylation, amidation, derivatization by known protecting/blocking

groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, etc. Additionally, the analog or derivative may contain one or more non-classical amino acids.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the CAPIs of the invention, *e.g.*, for imaging these proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc.

## 5.11 Expression Of Antibodies

The antibodies of the invention can be produced by any suitable method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or by recombinant expression, and are preferably produced by recombinant expression technique.

Recombinant expression of antibodies, or fragments, derivatives or analogs thereof, requires construction of a nucleic acid that encodes the antibody. If the nucleotide sequence of the antibody is known, a nucleic acid encoding the antibody may be assembled from chemically synthesized oligonucleotides (*e.g.*, as described in Kutmeier et al., 1994, BioTechniques 17:242), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding antibody, annealing and ligation of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

Alternatively, the nucleic acid encoding the antibody may be obtained by cloning the antibody. If a clone containing the nucleic acid encoding the particular antibody is not available, but the sequence of the antibody molecule is known, a nucleic acid encoding the antibody may be obtained from a suitable source (*e.g.*, an antibody cDNA library, or cDNA library generated from any tissue or cells expressing the antibody) by PCR amplification using synthetic primers hybridizable to the 3′ and

5′ ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence..

If an antibody molecule that specifically recognizes a particular antigen is not available (or a source for a cDNA library for cloning a nucleic acid encoding such an antibody), antibodies specific for a particular antigen may be generated by any method known in the art, for example, by immunizing an animal, such as a rabbit, to generate polyclonal antibodies or, more preferably, by generating monoclonal antibodies. Alternatively, a clone encoding at least the Fab portion of the antibody may be obtained by screening Fab expression libraries (*e.g.*, as described in Huse et al., 1989, Science 246:1275-1281) for clones of Fab fragments that bind the specific antigen or by screening antibody libraries (See, *e.g.*, Clackson et al., 1991, Nature 352:624; Hane et al., 1997 Proc. Natl. Acad. Sci. USA 94:4937).

Once a nucleic acid encoding at least the variable domain of the antibody molecule is obtained, it may be introduced into a vector containing the nucleotide sequence encoding the constant region of the antibody molecule (see, *e.g.*, PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Patent No. 5,122,464). Vectors containing the complete light or heavy chain for co-expression with the nucleic acid to allow the expression of a complete antibody molecule are also available. Then, the nucleic acid encoding the antibody can be used to introduce the nucleotide substitution(s) or deletion(s) necessary to substitute (or delete) the one or more variable region cysteine residues participating in an intrachain disulfide bond with an amino acid residue that does not contain a sulfhydyl group. Such modifications can be carried out by any method known in the art for the introduction of specific mutations or deletions in a nucleotide sequence, for example, but not limited to, chemical mutagenesis, in vitro site directed mutagenesis (Hutchinson et al., 1978, J. Biol. Chem. 253:6551), PCT based methods, etc.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. 81:851-855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing genes from

a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. As described supra, a chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human antibody constant region, *e.g.*, humanized antibodies.

Once a nucleic acid encoding an antibody molecule of the invention has been obtained, the vector for the production of the antibody molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing the protein of the invention by expressing nucleic acid containing the antibody molecule sequences are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing an antibody molecule coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. See, for example, the techniques described in Sambrook et al. (1990, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and Ausubel et al. (eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY).

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce an antibody of the invention.

The host cells used to express a recombinant antibody of the invention may be either bacterial cells such as *Escherichia coli*, or, preferably, eukaryotic cells, especially for the expression of whole recombinant antibody molecule. In particular, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for antibodies (Foecking et al., 198, Gene 45:101; Cockett et al., 1990, Bio/Technology 8:2).

A variety of host-expression vector systems may be utilized to express an antibody molecule of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express the antibody molecule of the invention in situ. These include but are not limited to microorganisms such as bacteria (*e.g.*, *E. coli, B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast (*e.g.*, *Saccharomyces, Pichia*) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing antibody coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, HEK293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions comprising an antibody molecule, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In

general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The antibody coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). In mammalian host cells, a number of viral-based expression systems (*e.g.*, an adenovirus expression system) may be utilized.

As discussed above, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein.

For long-term, high-yield production of recombinant antibodies, stable expression is preferred. For example, cell lines that stably express an antibody of interest can be produced by transfecting the cells with an expression vector comprising the nucleotide sequence of the antibody and the nucleotide sequence of a selectable (*e.g.*, neomycin or hygromycin), and selecting for expression of the selectable marker. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the antibody molecule.

The expression levels of the antibody molecule can be increased by vector amplification (for a review, see Bebbington and Hentschel, The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning, Vol.3. (Academic Press, New York, 1987)). When a marker in the vector system expressing antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the

amplified region is associated with the antibody gene, production of the antibody will also increase (Crouse et al., 1983, Mol. Cell. Biol. 3:257).

The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, 1986, Nature 322:52; Kohler, 1980, Proc. Natl. Acad. Sci. USA 77:2197). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

Once the antibody molecule of the invention has been recombinantly expressed, it may be purified by any method known in the art for purification of an antibody molecule, for example, by chromatography (*e.g.*, ion exchange chromatography, affinity chromatography such as with protein A or specific antigen, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

Alternatively, any fusion protein may be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht et al., 1991, Proc. Natl. Acad. Sci. USA 88:8972-897). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the open reading frame of the gene is translationally fused to an amino-terminal tag consisting of six histidine residues. The tag serves as a matrix binding domain for the fusion protein. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni2+ nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

## 5.12 Conjugated Antibodies

In a preferred embodiment, anti-CAPI antibodies or fragments thereof are conjugated to a diagnostic or therapeutic moiety. The antibodies can be used for diagnosis or to determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive nuclides, positron emitting metals (for use in positron emission tomography), and nonradioactive paramagnetic metal ions. See generally U.S. Patent No. 4,741,900 for metal ions that can be conjugated to antibodies for use as diagnostics according to the present invention. Suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; suitable prosthetic groups include streptavidin, avidin and biotin; suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride and phycoerythrin; suitable luminescent materials include luminol; suitable bioluminescent materials include luciferase, luciferin, and aequorin; and suitable radioactive nuclides include 125I, 131I, 111In and 99Tc.

An anti-CAPI antibodies or fragments thereof can be conjugated to a therapeutic agent or drug moiety to modify a given biological response. The therapeutic agent or drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, α-interferon, β-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, a thrombotic agent or an anti-angiogenic agent, *e.g.*, angiostatin or endostatin; or, a biological response modifier such as a lymphokine, interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), granulocyte macrophage colony stimulating factor (GM-tissue), granulocyte colony stimulating factor (G-CSF), nerve growth factor (NGF) or other growth factor.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, *e.g.*, Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982).

Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980.

An antibody with or without a therapeutic moiety conjugated to it can be used as a therapeutic that is administered alone or in combination with cytotoxic factor(s) and/or cytokine(s).

## 5.13 Diagnosis and/or monitoring of chronic asthma

In accordance with the present invention, test samples of, tissue, serum, plasma or urine obtained from a subject suspected of having or known to have chronic asthma can be used for diagnosis or monitoring. In one embodiment, an altered abundance of one or more CAFs or CAPIs (or any combination of them) in a test sample relative to a control sample (from a subject or subjects free from chronic asthma) or a previously determined reference range indicates the presence of an early or late asthmatic response; CAFs and CAPIs suitable for this purpose are identified in Tables I and III, respectively, as described in detail above. In another embodiment, the relative abundance of one or more CAFs or CAPIs (or any combination of them) in

a test sample compared to a control sample or a previously determined reference range indicates a subtype of chronic asthma (*e.g.*, familial or sporadic chronic asthma). In yet another embodiment, the relative abundance of one or more CAFs or CAPIs (or any combination of them) in a test sample relative to a control sample or a previously determined reference range indicates the degree or severity of chronic asthma. In yet another embodiment, the relative abundance of one or more CAFs or CAPIs (or any combination of them) in a test sample relative to a control sample or a previously determined reference range indicates the presence of an asthmatic response that is responsive to asthma-related therapy. In any of the aforesaid methods, detection of one or more CAPIs described herein may optionally be combined with detection of one or more additional biomarkers for chronic asthma. Any suitable method in the art can be employed to measure the level of CAFs and CAPIs, including but not limited to the Preferred Technology described herein, kinase assays, immunoassays to detect and/or visualize the CAPI (*e.g.*, Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.). In cases where a CAPI has a known function, an assay for that function may be used to measure CAPI expression. In a further embodiment, an increased or decreased abundance of mRNA including one or more CAPIs identified in Table III (or any combination of them) in a test sample relative to a control sample or a previously determined reference range indicates the presence of chronic asthma. Any suitable hybridization assay can be used to detect CAPI expression by detecting and/or visualizing mRNA encoding the CAPI (*e.g.*, Northern assays, dot blots, in situ hybridization, etc.).

In another embodiment of the invention, labeled antibodies, derivatives and analogs thereof, which specifically bind to a CAPI, can be used for diagnostic purposes to detect, diagnose, or monitor chronic asthma. Preferably, chronic asthma is detected in an animal, more preferably in a mammal and most preferably in a human.

## 5.14 Screening Assays

The invention provides methods for identifying agents (*e.g.*, candidate compounds or test compounds) that bind to a CAPI or have a stimulatory or inhibitory effect on the expression or activity of a CAPI. Preferably the CAPI is one of: CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38. The invention also provides methods of identifying agents, candidate compounds or test compounds that bind to a CAPI fragment, a CAPI-related polypeptide or a CAPI fusion protein or have a stimulatory or inhibitory effect on the expression or activity of a CAPI fragment, a CAPI-related polypeptide or a CAPI fusion protein.

Examples of agents, candidate compounds or test compounds include, but are not limited to, nucleic acids (*e.g.*, DNA and RNA), carbohydrates, lipids, proteins, peptides, peptidomimetics, small molecules and other drugs. Agents can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, 1997, Anticancer Drug Des. 12:145; U.S. Patent No. 5,738,996; and U.S. Patent No.5,807,683, each of which is incorporated herein in its entirety by reference).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al., 1993, Proc. Natl. Acad. Sci. USA 90:6909; Erb et al., 1994, Proc. Natl. Acad. Sci. USA 91:11422; Zuckermann et al., 1994, J. Med. Chem. 37:2678; Cho et al., 1993, Science 261:1303; Carrell et al., 1994, Angew. Chem. Int. Ed. Engl. 33:2059; Carell et al., 1994, Angew. Chem. Int. Ed. Engl. 33:2061; and Gallop et al., 1994, J. Med. Chem. 37:1233, each of which is incorporated herein in its entirety by reference.

Libraries of compounds may be presented, *e.g.*, presented in solution (*e.g.*, Houghten, 1992, Bio/Techniques 13:412-421), or on beads (Lam, 1991, Nature 354:82-84), chips (Fodor, 1993, Nature 364:555-556), bacteria (U.S. Patent No. 5,223,409), spores (Patent Nos. 5,571,698; 5,403,484; and 5,223,409), plasmids (Cull et al., 1992, Proc. Natl. Acad. Sci. USA 89:1865-1869) or phage (Scott and Smith, 1990, Science 249:386-390; Devlin, 1990, Science 249:404-406; Cwirla et al., 1990, Proc. Natl. Acad. Sci. USA 87:6378-6382; and Felici, 1991, J. Mol. Biol. 222:301-310), each of which is incorporated herein in its entirety by reference.

In one embodiment, agents that interact with (*i.e.*, bind to) a CAPI, a CAPI fragment (*e.g.* a functionally active fragment), a CAPI-related polypeptide, a fragment of a CAPI-related polypeptide, or a CAPI fusion protein are identified in a cell-based assay system. In accordance with this embodiment, cells expressing a CAPI, a fragment of a CAPI, a CAPI-related polypeptide, a fragment of a CAPI-related polypeptide, or a CAPI fusion protein are contacted with a candidate compound or a control compound and the ability of the candidate compound to interact with the CAPI is determined. If desired, this assay may be used to screen a plurality (*e.g.* a library) of candidate compounds. The cell, for example, can be of prokaryotic origin (*e.g.*, E. coli) or eukaryotic origin (*e.g.*, yeast or mammalian). Further, the cells can express the CAPI, fragment of the CAPI, CAPI-related polypeptide, a fragment of the CAPI-related polypeptide, or a CAPI fusion protein endogenously or be genetically engineered to express the CAPI, fragment of the CAPI, CAPI-related polypeptide, a fragment of the CAPI-related polypeptide, or a CAPI fusion protein. In certain instances, the CAPI, fragment of the CAPI, CAPI-related polypeptide, a fragment of the CAPI-related polypeptide, or a CAPI fusion protein or the candidate compound is labeled, for example with a radioactive label (such as $^{32}P$, $^{35}S$ or $^{125}I$) or a fluorescent label (such as fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde or fluorescamine) to enable detection of an interaction between a CAPI and a candidate compound. The ability of the candidate compound to interact directly or indirectly with a CAPI, a fragment of a CAPI, a CAPI-related polypeptide, a fragment of a CAPI-related polypeptide, or a CAPI fusion

protein can be determined by methods known to those of skill in the art. For example, the interaction between a candidate compound and a CAPI, a fragment of a CAPI, a CAPI-related polypeptide, a fragment of a CAPI-related polypeptide, or a CAPI fusion protein can be determined by flow cytometry, a scintillation assay, immunoprecipitation or western blot analysis.

In another embodiment, agents that interact with (*i.e.*, bind to) a CAPI, a CAPI fragment (*e.g.*, a functionally active fragment) a CAPI-related polypeptide, a fragment of a CAPI-related polypeptide, or a CAPI fusion protein are identified in a cell-free assay system. In accordance with this embodiment, a native or recombinant CAPI or fragment thereof, or a native or recombinant CAPI-related polypeptide or fragment thereof, or a CAPI-fusion protein or fragment thereof, is contacted with a candidate compound or a control compound and the ability of the candidate compound to interact with the CAPI or CAPI-related polypeptide, or CAPI fusion protein is determined. If desired, this assay may be used to screen a plurality (*e.g.* a library) of candidate compounds. Preferably, the CAPI, CAPI fragment, CAPI-related polypeptide, a fragment of a CAPI-related polypeptide, or a CAPI-fusion protein is first immobilized, by, for example, contacting the CAPI, CAPI fragment, CAPI-related polypeptide, a fragment of a CAPI-related polypeptide, or a CAPI fusion protein with an immobilized antibody which specifically recognizes and binds it, or by contacting a purified preparation of the CAPI, CAPI fragment, CAPI-related polypeptide, fragment of a CAPI-related polypeptide, or a CAPI fusion protein with a surface designed to bind proteins. The CAPI, CAPI fragment, CAPI-related polypeptide, a fragment of a CAPI-related polypeptide, or a CAPI fusion protein may be partially or completely purified (*e.g.*, partially or completely free of other polypeptides) or part of a cell lysate. Further, the CAPI, CAPI fragment, CAPI-related polypeptide, a fragment of a CAPI-related polypeptide may be a fusion protein comprising the CAPI or a biologically active portion thereof, or CAPI-related polypeptide and a domain such as glutathionine-S-transferase. Alternatively, the CAPI, CAPI fragment, CAPI-related polypeptide, fragment of a CAPI-related polypeptide or CAPI fusion protein can be biotinylated using techniques well known

to those of skill in the art (*e.g.*, biotinylation kit, Pierce Chemicals; Rockford, IL). The ability of the candidate compound to interact with a CAPI, CAPI fragment, CAPI-related polypeptide, a fragment of a CAPI-related polypeptide, or a CAPI fusion protein can be can be determined by methods known to those of skill in the art.

In another embodiment, a cell-based assay system is used to identify agents that bind to or modulate the activity of a protein, such as an enzyme, or a biologically active portion thereof, which is responsible for the production or degradation of a CAPI or is responsible for the post- translational modification of a CAPI. In a primary screen, a plurality (*e.g.*, a library) of compounds are contacted with cells that naturally or recombinantly express: (i) a CAPI, an isoform of a CAPI, a CAPI homolog a CAPI-related polypeptide, a CAPI fusion protein, or a biologically active fragment of any of the foregoing; and (ii) a protein that is responsible for processing of the CAPI, CAPI isoform, CAPI homolog, CAPI-related polypeptide, CAPI fusion protein, or fragment in order to identify compounds that modulate the production, degradation, or post-translational modification of the CAPI, CAPI isoform, CAPI homolog, CAPI-related polypeptide, CAPI fusion protein or fragment. If desired, compounds identified in the primary screen can then be assayed in a secondary screen against cells naturally or recombinantly expressing the specific CAPI of interest. The ability of the candidate compound to modulate the production, degradation or post-translational modification of a CAPI, isoform, homolog, CAPI-related polypeptide, or CAPI fusion protein can be determined by methods known to those of skill in the art, including without limitation, flow cytometry, a scintillation assay, immunoprecipitation and western blot analysis.

In another embodiment, agents that competitively interact with (*i.e.*, bind to) a CAPI, CAPI fragment, CAPI-related polypeptide, a fragment of a CAPI-related polypeptide, or a CAPI fusion protein are identified in a competitive binding assay. In accordance with this embodiment, cells expressing a CAPI, CAPI fragment, CAPI-related polypeptide, a fragment of a CAPI-related polypeptide, or a CAPI fusion protein are contacted with a candidate compound and a compound known to interact with the CAPI, CAPI fragment, CAPI-related polypeptide, a fragment of a CAPI-

related polypeptide or a CAPI fusion protein; the ability of the candidate compound to competitively interact with the CAPI, CAPI fragment, CAPI-related polypeptide, fragment of a CAPI-related polypeptide, or a CAPI fusion protein is then determined. Alternatively, agents that competitively interact with (*i.e.*, bind to) a CAPI, CAPI fragment, CAPI-related polypeptide or fragment of a CAPI-related polypeptide are identified in a cell-free assay system by contacting a CAPI, CAPI fragment, CAPI-related polypeptide, fragment of a CAPI-related polypeptide, or a CAPI fusion protein with a candidate compound and a compound known to interact with the CAPI, CAPI-related polypeptide or CAPI fusion protein. As stated above, the ability of the candidate compound to interact with a CAPI, CAPI fragment, CAPI-related polypeptide, a fragment of a CAPI-related polypeptide, or a CAPI fusion protein can be determined by methods known to those of skill in the art. These assays, whether cell-based or cell-free, can be used to screen a plurality (*e.g.*, a library) of candidate compounds.

In another embodiment, agents that modulate (*i.e.*, upregulate or downregulate) the expression of a CAPI, or a CAPI-related polypeptide are identified by contacting cells (*e.g.*, cells of prokaryotic origin or eukaryotic origin) expressing the CAPI, or CAPI-related polypeptide with a candidate compound or a control compound (*e.g.*, phosphate buffered saline (PBS)) and determining the expression of the CAPI, CAPI-related polypeptide, or CAPI fusion protein, mRNA encoding the CAPI, or mRNA encoding the CAPI-related polypeptide. The level of expression of a selected CAPI, CAPI-related polypeptide, mRNA encoding the CAPI, or mRNA encoding the CAPI-related polypeptide in the presence of the candidate compound is compared to the level of expression of the CAPI, CAPI-related polypeptide, mRNA encoding the CAPI, or mRNA encoding the CAPI-related polypeptide in the absence of the candidate compound (*e.g.*, in the presence of a control compound). The candidate compound can then be identified as a modulator of the expression of the CAPI, or a CAPI-related polypeptide based on this comparison. For example, when expression of the CAPI or mRNA is significantly greater in the presence of the candidate compound than in its absence, the candidate compound is identified as a

stimulator of expression of the CAPI or mRNA. Alternatively, when expression of the CAPI or mRNA is significantly less in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of the expression of the CAPI or mRNA. The level of expression of a CAPI or the mRNA that encodes it can be determined by methods known to those of skill in the art. For example, mRNA expression can be assessed by Northern blot analysis or RT-PCR, and protein levels can be assessed by western blot analysis.

In another embodiment, agents that modulate the activity of a CAPI, or a CAPI-related polypeptide are identified by contacting a preparation containing the CAPI or CAPI-related polypeptide, or cells (*e.g.*, prokaryotic or eukaryotic cells) expressing the CAPI or CAPI-related polypeptide with a test compound or a control compound and determining the ability of the test compound to modulate (*e.g.*, stimulate or inhibit) the activity of the CAPI or CAPI-related polypeptide. The activity of a CAPI or a CAPI-related polypeptide can be assessed by detecting induction of a cellular signal transduction pathway of the CAPI or CAPI-related polypeptide (*e.g.*, intracellular Ca2+, diacylglycerol, IP3, etc.), detecting catalytic or enzymatic activity of the target on a suitable substrate, detecting the induction of a reporter gene (e.g., a regulatory element that is responsive to a CAPI or a CAPI-related polypeptide and is operably linked to a nucleic acid encoding a detectable marker, *e.g.*, luciferase), or detecting a cellular response, for example, cellular differentiation, or cell proliferation. Based on the present description, techniques known to those of skill in the art can be used for measuring these activities (see, *e.g.*, U.S. Patent No. 5,401,639, which is incorporated herein by reference). The candidate compound can then be identified as a modulator of the activity of a CAPI or CAPI-related polypeptide by comparing the effects of the candidate compound to the control compound. Suitable control compounds include phosphate buffered saline (PBS) and normal saline (NS).

In another embodiment, agents that modulate (*i.e.*, upregulate or downregulate) the expression, activity or both the expression and activity of a CAPI or CAPI-related polypeptide are identified in an animal model. Examples of suitable

animals include, but are not limited to, mice, rats, rabbits, monkeys, guinea pigs, dogs and cats. Preferably, the animal used represents a model of chronic asthma (*e.g.*, the ovalbumin-induced allergic airway disease model (Fiscus, L. C., J. Van Herpen, et al. (2001). "L-Selectin is required for the development of airway hyperresponsiveness but not airway inflammation in a murine model of asthma." J Allergy Clin Immunol 107(6): 1019-24; Sato, Y., T. Kishi, et al. (1998). "Histopathological and immunohistochemical studies on experimental asthmatic model induced by aerosolised ovalbumin inhalation in guinea pigs." J Toxicol Sci 23(1): 69-75; Kamachi, A., M. Munakata, et al. (2001). "Enhancement of goblet cell hyperplasia and airway hyperresponsiveness by salbutamol in a rat model of atopic asthma." Thorax 56(1): 19-24), BALB/c mice (Martin, R. J., H. W. Chu, et al. (2001). "Airway inflammation and bronchial hyperresponsiveness after Mycoplasma pneumoniae infection in a murine model." Am J Respir Cell Mol Biol 24(5): 577-82). In accordance with this embodiment, the test compound or a control compound is administered (*e.g.*, orally, rectally or parenterally such as intraperitoneally or intravenously) to a suitable animal and the effect on the expression, activity or both expression and activity of the CAPI or CAPI-related polypeptide is determined. Changes in the expression of a CAPI or CAPI-related polypeptide can be assessed by the methods outlined above.

In yet another embodiment, a CAPI or CAPI-related polypeptide is used as a "bait protein" in a two-hybrid assay or three hybrid assay to identify other proteins that bind to or interact with a CAPI or CAPI-related polypeptide (see, *e.g.*, U.S. Patent No. 5,283,317; Zervos et al. (1993) Cell 72:223-232; Madura et al. (1993) J. Biol. Chem. 268:12046-12054; Bartel et al. (1993) Bio/Techniques 14:920-924; Iwabuchi et al. (1993) Oncogene 8:1693-1696; and PCT Publication No. WO 94/10300). As those skilled in the art will appreciate, such binding proteins are also likely to be involved in the propagation of signals by the CAPIs of the invention as, for example, upstream or downstream elements of a signaling pathway involving the CAPIs of the invention.

Table VII enumerates scientific publications describing suitable assays for detecting or quantifying enzymatic or binding activity of a CAPI, a CAPI analog, a

CAPI-related polypeptide, or a fragment of any of the foregoing. Each such reference is hereby incorporated in its entirety. In a preferred embodiment, an assay referenced in Table XVII is used in the screens and assays described herein, for example to screen for or identify a compound that modulates the activity of (or that modulates both the expression and activity of) a CAPI, CAPI analog, or CAPI-related polypeptide, a fragment of any of the foregoing or a CAPI fusion protein.

Table VII: References describing suitable assays for CAPIs

Table VII

| CAF# | Protein Name | Assay reference |
|---|---|---|
| CAF-5 | HEAT SHOCK PROTEIN HSP 90-BETA (HSP 84) (HSP 90) | " Hsp90 Chaperone Activity Requires the Full-length Protein and Interaction among Its Multiple Domains" (2000) J. Biol. Chem., 275(42), pp32499-32507. |
| CAF-12 | PHOSPHOGLUCOMUTASE (EC 5.4.2.2) (GLUCOSE PHOSPHOMUTASE) (PGM) | " Purification and partial characterization of the phosphoglucomutase isozymes from human placenta" (1990), Prep. Biochem. 20(3-4), pp219-240. " Isoenzymes of human red blood cells: isolation and kinetic properties", (1989), Prep Biochem. 19(3), pp251-271 "Human erythrocyte phosphoglucomutase: comparison of the properties of PGM1 and PGM2 isoenzymes" (1984), Biochimie, 66(9-10), pp617-623 "The effect of storage upon the activity of phosphoglucomutase and adenylate kinase enzymes" (1970), Med Sci Law, 10(4), pp230-4 |
| CAF-19 | GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE, LIVER (EC 1.2.1.12) | "Alteration of glyceraldehyde-3-phosphate dehydrogenase activity and messenger mRNA content by androgen in human prostate carcinoma cells", (1995), Cancer |

Table VII

| GAR# | Protein Name | Assay reference |
|---|---|---|
| | | Res. 55(19, pp4234-6 "increased membrane activity of glyceraldehyde 3-phosphate dehydrogenase in erythrocytes of patients with homozygous sickle cell anaemia", (1992), Clin Chim Acta 209(3), pp189-95 "Heterogeneity of glyceraldehyde-3-phosphate dehydrogenase from human brain" (1988), 954(3), pp309-24 |

This invention further provides novel agents identified by the above-described screening assays and uses thereof for treatments as described herein.

## 5.15 Therapeutic Uses of CAPIs

The invention provides for treatment or prevention of various diseases and disorders by administration of a therapeutic compound. Such compounds include but are not limited to: CAPIs, CAPI analogs, CAPI-related polypeptides and derivatives (including fragments) thereof; antibodies to the foregoing; nucleic acids encoding CAPIs, CAPI analogs, CAPI-related polypeptides and fragments thereof; antisense nucleic acids to a gene encoding a CAPI or CAPI-related polypeptide; and modulator (*e.g.*, agonists and antagonists) of a gene encoding a CAPI or CAPI-related polypeptide. An important feature of the present invention is the identification of genes encoding CAPIs involved in chronic asthma. Chronic asthma can be treated (*e.g.*, to ameliorate symptoms or to retard onset or progression) or prevented by administration of a therapeutic compound that promotes function or expression of one or more CAPIs that are decreased in the tissue of chronic asthma subjects having chronic asthma, or by administration of a therapeutic compound that reduces function or expression of one or more CAPIs that are increased in the tissue of subjects having chronic asthma.

In one embodiment, one or more antibodies each specifically binding to a CAPI are administered alone or in combination with one or more additional therapeutic compounds or treatments. Examples of such therapeutic compounds or treatments include, but are not limited to, glucocorticoids (dexamethasone) and phosphodiesterase inhibitors (theophylline).

Preferably, a biological product such as an antibody is allogeneic to the subject to which it is administered. In a preferred embodiment, a human CAPI or a human CAPI-related polypeptide, a nucleotide sequence encoding a human CAPI or a human CAPI-related polypeptide, or an antibody to a human CAPI or a human CAPI-related polypeptide, is administered to a human subject for therapy (*e.g.* to ameliorate symptoms or to retard onset or progression) or prophylaxis.

5.15.1 Treatment And Prevention Of Chronic Asthma

Chronic asthma can be treated or prevented by administration to a subject suspected of having or known to have chronic asthma or to be at risk of developing chronic asthma of a compound that modulates (*i.e.*, increases or decreases) the level or activity (*i.e.*, function) of one or more CAPIs -- or the level of one or more CAFs -- that are differentially present in the tissue of subjects having chronic asthma compared with tissue of subjects free from asthma. In one embodiment, chronic asthma is treated or prevented by administering to a subject suspected of having or known to have chronic asthma or to be at risk of developing chronic asthma a compound that upregulates (*i.e.*, increases) the level or activity (*i.e.*, function) of one or more CAPIs - or the level of one or more CAFs -- that are decreased in the tissue of subjects having chronic asthma. In another embodiment, a compound is administered that upregulates the level or activity (*i.e.*, function) of one or more CAPIs -- or the level of one or more CAFs -- that are increased in the tissue of subjects having chronic asthma. Examples of such a compound include but are not limited to: CAPIs, CAPI fragments and CAPI-related polypeptides; nucleic acids encoding a CAPI, a CAPI fragment and a CAPI-related polypeptide (*e.g.*, for use in gene therapy); and, for those CAPIs or CAPI-related polypeptides with enzymatic activity, compounds or molecules known to

modulate that enzymatic activity. Other compounds that can be used, *e.g.*, CAPI agonists, can be identified using in vitro assays.

Chronic asthma is also treated or prevented by administration to a subject suspected of having or known to have chronic asthma or to be at risk of developing chronic asthma of a compound that downregulates the level or activity of one or more CAPIs - or the level of one or more CAFs - that are increased in the tissue of subjects having chronic asthma. In another embodiment, a compound is administered that downregulates the level or activity of one or more CAPIs - or the level of one or more CAFs - that are decreased in the tissue of subjects having chronic asthma. Examples of such a compound include, but are not limited to, CAPI antisense oligonucleotides, ribozymes, antibodies directed against CAPIs, and compounds that inhibit the enzymatic activity of a CAPI. Other useful compounds *e.g.*, CAPI antagonists and small molecule CAPI antagonists, can be identified using in vitro assays.

In a preferred embodiment, therapy or prophylaxis is tailored to the needs of an individual subject. Thus, in specific embodiments, compounds that promote the level or function of one or more CAPIs, or the level of one or more CAFs, are therapeutically or prophylactically administered to a subject suspected of having or known to have chronic asthma, in whom the levels or functions of said one or more CAPIs, or levels of said one or more CAFs, are absent or are decreased relative to a control or normal reference range. In further embodiments, compounds that promote the level or function of one or more CAPIs, or the level of one or more CAFs, are therapeutically or prophylactically administered to a subject suspected of having or known to have chronic asthma in whom the levels or functions of said one or more CAPIs, or levels of said one or more CAFs, are increased relative to a control or to a reference range. In further embodiments, compounds that decrease the level or function of one or more CAPIs, or the level of one or more CAFs, are therapeutically or prophylactically administered to a subject suspected of having or known to have chronic asthma in whom the levels or functions of said one or more CAPIs, or levels of said one or more CAFs, are increased relative to a control or to a reference range. In further embodiments, compounds that decrease the level or function of one or more

CAPIs, or the level of one or more CAFs, are therapeutically or prophylactically administered to a subject suspected of having or known to have chronic asthma in whom the levels or functions of said one or more CAPIs, or levels of said one or more CAFs, are decreased relative to a control or to a reference range. The change in CAPI function or level, or CAF level, due to the administration of such compounds can be readily detected, *e.g.*, by obtaining a sample (*e.g.*, a tissue sample such as biopsy tissue or a sample of upper airways, blood or urine) and assaying in vitro the levels of said CAFs or the levels or activities of said CAPIs, or the levels of mRNAs encoding said CAPIs. or any combination of the foregoing. Such assays can be performed before and after the administration of the compound as described herein.

The compounds of the invention include but are not limited to any compound, *e.g.*, a small organic molecule, protein, peptide, antibody, nucleic acid, etc. that restores the chronic asthma CAPI or CAF profile towards normal.

5.15.2 Gene Therapy

In a specific embodiment, nucleic acids comprising a sequence encoding a CAPI, a CAPI fragment, CAPI-related polypeptide or fragment of a CAPI-related polypeptide, are administered to promote CAPI function by way of gene therapy. Gene therapy refers to administration to a subject of an expressed or expressible nucleic acid. In this embodiment, the nucleic acid produces its encoded polypeptide that mediates a therapeutic effect by promoting CAPI function.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 11(5): 155-215). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.),

1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a particular aspect, the compound comprises a nucleic acid encoding a CAPI or fragment or chimeric protein thereof, said nucleic acid being part of an expression vector that expresses a CAPI or fragment or chimeric protein thereof in a suitable host. In particular, such a nucleic acid has a promoter operably linked to the CAPI coding region, said promoter being inducible or constitutive (and, optionally, tissue-specific). In another particular embodiment, a nucleic acid molecule is used in which the CAPI coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the CAPI nucleic acid (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

Delivery of the nucleic acid into a subject may be direct, in which case the subject is directly exposed to the nucleic acid or nucleic acid-carrying vector; this approach is known as in vivo gene therapy. Alternatively, delivery of the nucleic acid into the subject may be indirect, in which case cells are first transformed with the nucleic acid in vitro and then transplanted into the subject; this approach is known as ex vivo gene therapy.

In another embodiment, the nucleic acid is directly administered in vivo, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286); by direct injection of naked DNA; by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont); by coating with lipids, cell-surface receptors or transfecting agents; by encapsulation in liposomes, microparticles or microcapsules; by administering it in linkage to a peptide which is known to enter the nucleus; or by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-

4432), which can be used to target cell types specifically expressing the receptors. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted in vivo for cell specific uptake and expression, by targeting a specific receptor (see, *e.g.*, PCT Publications WO 92/06180 dated April 16, 1992 (Wu et al.); WO 92/22635 dated December 23, 1992 (Wilson et al.); WO92/20316 dated November 26, 1992 (Findeis et al.); WO93/14188 dated July 22, 1993 (Clarke et al.), WO 93/20221 dated October 14, 1993 (Young)). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

In a further embodiment, a viral vector that contains a nucleic acid encoding a CAPI is used. For example, a retroviral vector can be used (see Miller et al., 1993, Meth. Enzymol. 217:581-599). These retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The nucleic acid encoding the CAPI to be used in gene therapy is cloned into the vector, which facilitates delivery of the gene into a subject. More detail about retroviral vectors can be found in Boesen et al., 1994, Biotherapy 6:291-302, which describes the use of a retroviral vector to deliver the mdr1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., 1994, J. Clin. Invest. 93:644-651; Kiem et al., 1994, Blood 83:1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114.

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of

being capable of infecting non-dividing cells. Kozarsky and Wilson, 1993, Current Opinion in Genetics and Development 3:499-503 present a review of adenovirus-based gene therapy. Bout et al., 1994, Human Gene Therapy 5:3-10 demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., 1991, Science 252:431-434; Rosenfeld et al., 1992, Cell 68:143-155; Mastrangeli et al., 1993, J. Clin. Invest. 91:225-234; PCT Publication WO94/12649; and Wang, et al., 1995, Gene Therapy 2:775-783.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., 1993, Proc. Soc. Exp. Biol. Med. 204:289-300; U.S. Patent No. 5,436,146).

Another suitable approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a subject.

In this embodiment, the nucleic acid is introduced into a cell prior to administration in vivo of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see, *e.g.*, Loeffler and Behr, 1993, Meth. Enzymol. 217:599-618; Cohen et al., 1993, Meth. Enzymol. 217:618-644; Cline, 1985, Pharmac. Ther. 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a subject by various methods known in the art. In a preferred embodiment, epithelial cells are injected, *e.g.*, subcutaneously. In another embodiment, recombinant skin cells may be applied as a skin graft onto the subject. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, the condition of the subject, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to neuronal cells, glial cells (*e.g.*, oligodendrocytes or astrocytes), epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood or fetal liver.

In a preferred embodiment, the cell used for gene therapy is autologous to the subject that is treated.

In an embodiment in which recombinant cells are used in gene therapy, a nucleic acid encoding a CAPI is introduced into the cells such that it is expressible by the cells or their progeny, and the recombinant cells are then administered in vivo for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem or progenitor cells which can be isolated and maintained in vitro can be used in accordance with this embodiment of the present invention (see *e.g.* PCT Publication WO 94/08598, dated April 28, 1994; Stemple and Anderson, 1992, Cell 71:973-985; Rheinwald, 1980, Meth. Cell Bio. 21A:229; and Pittelkow and Scott, 1986, Mayo Clinic Proc. 61:771).

In another embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

Direct injection of a DNA coding for a CAPI may also be performed according to, for example, the techniques described in United States Patent No. 5,589,466. These techniques involve the injection of "naked DNA", *i.e.*, isolated DNA molecules in the absence of liposomes, cells, or any other material besides a suitable carrier. The injection of DNA encoding a protein and operably linked to a suitable promoter results in the production of the protein in cells near the site of injection and the elicitation of an immune response in the subject to the protein encoded by the injected DNA. In a preferred embodiment, naked DNA comprising (a) DNA encoding a CAPI and (b) a promoter are injected into a subject to elicit an immune response to the CAPI.

5.15.3 Inhibition of CAPIs To Treat Chronic Asthma

In one embodiment of the invention, chronic asthma is treated or prevented by administration of a compound that antagonizes (inhibits) the level(s) and/or function(s) of one or more CAPIs which are elevated in the tissue of subjects having chronic asthma as compared with tissue of subjects free from chronic asthma. Compounds useful for this purpose include but are not limited to anti-CAPI antibodies (and fragments and derivatives containing the binding region thereof), CAPI antisense or ribozyme nucleic acids, and nucleic acids encoding dysfunctional CAPIs that are used to "knockout" endogenous CAPI function by homologous recombination (see, *e.g.*, Capecchi, 1989, Science 244:1288-1292). Other compounds that inhibit CAPI function can be identified by use of known in vitro assays, *e.g.*, assays for the ability of a test compound to inhibit binding of a CAPI to another protein or a binding partner, or to inhibit a known CAPI function. Preferably such inhibition is assayed in vitro or in cell culture, but genetic assays may also be employed. The Preferred Technology can also be used to detect levels of the CAPI before and after the administration of the compound. Preferably, suitable in vitro or in vivo assays are utilized to determine the effect of a specific compound and whether its administration is indicated for treatment of the affected tissue, as described in more detail below.

In a particular embodiment, a compound that inhibits a CAPI function is administered therapeutically or prophylactically to a subject in whom an increased tissue level or functional activity of the CAPI (*e.g.*, greater than the normal level or desired level) is detected as compared with tissue of subjects free from chronic asthma or a predetermined reference range. Methods standard in the art can be employed to measure the increase in a CAPI level or function, as outlined above. Preferred CAPI inhibitor compositions include small molecules, *i.e.*, molecules of 1000 daltons or less. Such small molecules can be identified by the screening methods described herein.

5.15.4 Antisense Regulation of CAPIs

In a further embodiment, CAPI expression is inhibited by use of CAPI antisense nucleic acids. The present invention provides the therapeutic or prophylactic use of nucleic acids comprising at least six nucleotides that are antisense to a gene or cDNA encoding a CAPI or a portion thereof. As used herein, a CAPI "antisense" nucleic acid refers to a nucleic acid capable of hybridizing by virtue of some sequence complementarity to a portion of an RNA (preferably mRNA) encoding a CAPI. The antisense nucleic acid may be complementary to a coding and/or noncoding region of an mRNA encoding a CAPI. Such antisense nucleic acids have utility as compounds that inhibit CAPI expression, and can be used in the treatment or prevention of chronic asthma.

The antisense nucleic acids of the invention are double-stranded or single-stranded oligonucleotides, RNA or DNA or a modification or derivative thereof, and can be directly administered to a cell or produced intracellularly by transcription of exogenous, introduced sequences.

The invention further provides pharmaceutical compositions comprising an effective amount of the CAPI antisense nucleic acids of the invention in a pharmaceutically acceptable carrier, as described infra.

In another embodiment, the invention provides methods for inhibiting the expression of a CAPI nucleic acid sequence in a prokaryotic or eukaryotic cell

comprising providing the cell with an effective amount of a composition comprising a CAPI antisense nucleic acid of the invention,

CAPI antisense nucleic acids and their uses are described in detail below.

5.15.5 CAPI Antisense Nucleic Acids

The CAPI antisense nucleic acids are of at least six nucleotides and are preferably oligonucleotides ranging from 6 to about 50 oligonucleotides. In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 15 nucleotides, at least 100 nucleotides, or at least 200 nucleotides. The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof and can be single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appended groups such as peptides; agents that facilitate transport across the cell membrane (see, *e.g.*, Letsinger et al., 1989, Proc. Natl. Acad. Sci. USA 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. WO 88/09810, published December 15, 1988); hybridization-triggered cleavage agents (see, *e.g.*, Krol et al., 1988, BioTechniques 6:958-976) or intercalating agents (see, *e.g.*, Zon, 1988, Pharm. Res. 5:539-549).

In a particular aspect of the invention, a CAPI antisense oligonucleotide is provided, preferably of single-stranded DNA. The oligonucleotide may be modified at any position on its structure with substituents generally known in the art.

The CAPI antisense oligonucleotide may comprise at least one of the following modified base moieties: 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5□-methoxycarboxymethyluracil, 5-methoxyuracil, 2-

methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, 2,6-diaminopurine, and other base analogs.

In another embodiment, the oligonucleotide comprises at least one modified sugar moiety, *e.g.*, one of the following sugar moieties: arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the oligonucleotide comprises at least one of the following modified phosphate backbones: a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, a formacetal, or an analog of formacetal.

In yet another embodiment, the oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641).

The oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent.

Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. USA 85:7448-7451).

In another embodiment, the CAPI antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector can be introduced in vivo such that it is taken up by a cell, within which cell the vector or a portion thereof is transcribed, producing an antisense nucleic acid (RNA)

of the invention. Such a vector would contain a sequence encoding the CAPI antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the CAPI antisense RNA can be by any promoter known in the art to act in mammalian, preferably human, cells. Such promoters can be inducible or constitutive. Examples of such promoters are outlined above.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a gene encoding a CAPI, preferably a human gene encoding a CAPI. However, absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," as referred to herein, means a sequence having sufficient complementarity to be able to hybridize under stringent conditions (*e.g.*, highly stringent conditions comprising hybridization in 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C and washing in 0.1xSSC/0.1% SDS at 68°C, or moderately stringent conditions comprising washing in 0.2xSSC/0.1% SDS at 42°C ) with the RNA, forming a stable duplex; in the case of double-stranded CAPI antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA encoding a CAPI it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

5.15.6 Therapeutic Use of CAPI Antisense Nucleic Acids

The CAPI antisense nucleic acids can be used to treat or prevent chronic asthma when the target CAPI is overexpressed in the tissue of subjects suspected of having or suffering from chronic asthma. In a preferred embodiment, a single-stranded DNA antisense CAPI oligonucleotide is used.

Cell types that express or overexpress RNA encoding a CAPI can be identified by various methods known in the art. Such cell types include but are not limited to leukocytes (*e.g.*, neutrophils, macrophages, monocytes) and resident cells (*e.g.*, astrocytes, glial cells, neuronal cells, and ependymal cells). Such methods include, but are not limited to, hybridization with a CAPI-specific nucleic acid (*e.g.*, by Northern hybridization, dot blot hybridization, in situ hybridization), observing the ability of RNA from the cell type to be translated in vitro into a CAPI, immunoassay, etc. In a preferred aspect, primary tissue from a subject can be assayed for CAPI expression prior to treatment, *e.g.*, by immunocytochemistry or in situ hybridization.

Pharmaceutical compositions of the invention, comprising an effective amount of a CAPI antisense nucleic acid in a pharmaceutically acceptable carrier, can be administered to a subject having chronic asthma.

The amount of CAPI antisense nucleic acid which will be effective in the treatment of chronic asthma can be determined by standard clinical techniques.

In a specific embodiment, pharmaceutical compositions comprising one or more CAPI antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, such compositions may be used to achieve sustained release of the CAPI antisense nucleic acids.

5.15.7 Inhibitory Ribozyme And Triple Helix Approaches

In another embodiment, symptoms of chronic asthma may be ameliorated by decreasing the level of a CAPI or CAPI activity by using gene sequences encoding the CAPI in conjunction with well-known gene "knock-out," ribozyme or triple helix methods to decrease gene expression of a CAPI. In this approach ribozyme or triple helix molecules are used to modulate the activity, expression or synthesis of the gene

encoding the CAPI, and thus to ameliorate the symptoms of chronic asthma. Such molecules may be designed to reduce or inhibit expression of a mutant or non-mutant target gene. Techniques for the production and use of such molecules are well known to those of skill in the art.

Ribozyme molecules designed to catalytically cleave gene mRNA transcripts encoding a CAPI can be used to prevent translation of target gene mRNA and, therefore, expression of the gene product. (See, *e.g.*, PCT International Publication WO90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225).

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. (For a review, see Rossi, 1994, Current Biology 4, 469-471). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage event. The composition of ribozyme molecules must include one or more sequences complementary to the target gene mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see, *e.g.*, U.S. Patent No. 5,093,246, which is incorporated herein by reference in its entirety.

While ribozymes that cleave mRNA at site-specific recognition sequences can be used to destroy mRNAs encoding a CAPI, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5′-UG-3′. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Myers, 1995, Molecular Biology and Biotechnology: A Comprehensive Desk Reference, VCH Publishers, New York, (see especially Figure 4, page 833) and in Haseloff and Gerlach, 1988, Nature, 334, 585-591, each of which is incorporated herein by reference in its entirety.

Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5′ end of the mRNA encoding the CAPI, *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one that occurs naturally in Tetrahymena thermophila (known as the IVS, or L-19 IVS RNA) and that has been extensively described by Thomas Cech and collaborators (Zaug, et al., 1984, Science, 224, 574-578; Zaug and Cech, 1986, Science, 231, 470-475; Zaug, et al., 1986, Nature, 324, 429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been and Cech, 1986, Cell, 47, 207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in the gene encoding the CAPI.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.*, for improved stability, targeting, etc.) and should be delivered to cells that express the CAPI in vivo. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous mRNA encoding the CAPI and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficacy.

Endogenous CAPI expression can also be reduced by inactivating or "knocking out" the gene encoding the CAPI, or the promoter of such a gene, using targeted homologous recombination (*e.g.*, see Smithies, et al., 1985, Nature 317:230-234; Thomas and Capecchi, 1987, Cell 51:503-512; Thompson et al., 1989, Cell 5:313-321; and Zijlstra et al., 1989, Nature 342:435-438, each of which is incorporated by reference herein in its entirety). For example, a mutant gene encoding a non-functional CAPI (or a completely unrelated DNA sequence) flanked by DNA

homologous to the endogenous gene (either the coding regions or regulatory regions of the gene encoding the CAPI) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the target gene in vivo. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the target gene. Such approaches are particularly suited in the agricultural field where modifications to ES (embryonic stem) cells can be used to generate animal offspring with an inactive target gene (*e.g.*, see Thomas and Capecchi, 1987 and Thompson, 1989, supra). However this approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site in vivo using appropriate viral vectors.

Alternatively, the endogenous expression of a gene encoding a CAPI can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the gene (*i.e.*, the gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the gene encoding the CAPI in target cells in the body. (See generally, Helene, 1991, Anticancer Drug Des., 6(6), 569-584; Helene, et al., 1992, Ann. N.Y. Acad. Sci., 660, 27-36; and Maher, 1992, Bioassays 14(12), 807-815).

Nucleic acid molecules to be used in triplex helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC+ triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, contain a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

In instances wherein the antisense, ribozyme, or triple helix molecules described herein are utilized to inhibit mutant gene expression, it is possible that the technique may so efficiently reduce or inhibit the transcription (triple helix) or translation (antisense, ribozyme) of mRNA produced by normal gene alleles of a CAPI that the situation may arise wherein the concentration of CAPI present may be lower than is necessary for a normal phenotype. In such cases, to ensure that substantially normal levels of activity of a gene encoding a CAPI are maintained, gene therapy may be used to introduce into cells nucleic acid molecules that encode and express the CAPI that exhibit normal gene activity and that do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments are being utilized. Alternatively, in instances whereby the gene encodes an extracellular protein, normal CAPI can be co-administered in order to maintain the requisite level of CAPI activity.

Antisense RNA and DNA, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules, as discussed above. These include techniques for chemically synthesizing oligodeoxyri-bonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

## 5.16 Assays for Therapeutic r Prophylactic Comp unds

The present invention also provides assays for use in discovery of pharmaceutical products in order to identify or verify the efficacy of compounds for treatment or prevention of chronic asthma. Agents can be assayed for their ability to restore CAF or CAPI levels in a subject having chronic asthma towards levels found in subjects free from chronic asthma or to produce similar changes in experimental animal models of chronic asthma. Compounds able to restore CAF or CAPI levels in a subject having chronic asthma towards levels found in subjects free from chronic asthma or to produce similar changes in experimental animal models of chronic asthma can be used as lead compounds for further drug discovery, or used therapeutically. CAF and CAPI expression can be assayed by the Preferred Technology, immunoassays, gel electrophoresis followed by visualization, detection of CAPI activity, or any other method taught herein or known to those skilled in the art. Such assays can be used to screen candidate drugs, in clinical monitoring or in drug development, where abundance of a CAF or CAPI can serve as a surrogate marker for clinical disease.

Preferably the CAPI is selected from one of: CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38.

In various embodiments, in vitro assays can be carried out with cells representative of cell types involved in a subject's disorder, to determine if a compound has a desired effect upon such cell types.

Compounds for use in therapy can be tested in suitable animal model systems prior to testing in humans, including but not limited to rats, mice, chicken, cows, monkeys, rabbits, etc. For in vivo testing, prior to administration to humans, any animal model system known in the art may be used. It is also apparent to the skilled artisan that, based upon the present disclosure, transgenic animals can be produced with "knock-out" mutations of the gene or genes encoding one or more CAPIs. A

"knock-out" mutation of a gene is a mutation that causes the mutated gene to not be expressed, or expressed in an aberrant form or at a low level, such that the activity associated with the gene product is nearly or entirely absent. Preferably, the transgenic animal is a mammal, more preferably, the transgenic animal is a mouse.

In one embodiment, test compounds that modulate the expression of a CAPI are identified in non-human animals (*e.g.*, mice, rats, monkeys, rabbits, and guinea pigs), preferably non-human animal models for chronic asthma, expressing the CAPI. In accordance with this embodiment, a test compound or a control compound is administered to the animals, and the effect of the test compound on expression of one or more CAPIs is determined. A test compound that alters the expression of a CAPI (or a plurality of CAPIs) can be identified by comparing the level of the selected CAPI or CAPIs (or mRNA(s) encoding the same) in an animal or group of animals treated with a test compound with the level of the CAPI(s) or mRNA(s) in an animal or group of animals treated with a control compound. Techniques known to those of skill in the art can be used to determine the mRNA and protein levels, for example, in situ hybridization. The animals may or may not be sacrificed to assay the effects of a test compound.

In another embodiment, test compounds that modulate the activity of a CAPI or a biologically active portion thereof are identified in non-human animals (*e.g.*, mice, rats, monkeys, rabbits, and guinea pigs), preferably non-human animal models for chronic asthma, expressing the CAPI. In accordance with this embodiment, a test compound or a control compound is administered to the animals, and the effect of a test compound on the activity of a CAPI is determined. A test compound that alters the activity of a CAPI (or a plurality of CAPIs) can be identified by assaying animals treated with a control compound and animals treated with the test compound. The activity of the CAPI can be assessed by detecting induction of a cellular second messenger of the CAPI (*e.g.*, intracellular Ca2+, diacylglycerol, IP3, etc.), detecting catalytic or enzymatic activity of the CAPI or binding partner thereof, detecting the induction of a reporter gene (*e.g.*, a regulatory element that is responsive to a CAPI of the invention operably linked to a nucleic acid encoding a detectable marker, such as

luciferase or green fluorescent protein), or detecting a cellular response (*e.g.*, cellular differentiation or cell proliferation). Techniques known to those of skill in the art can be utilized to detect changes in the activity of a CAPI (see, *e.g.*, U.S. Patent No. 5,401,639, which is incorporated herein by reference).

In yet another embodiment, test compounds that modulate the level or expression of a CAPI (or plurality of CAPIs) are identified in human subjects having chronic asthma, preferably those having mild to severe chronic asthma and most preferably those having mild chronic asthma. In accordance with this embodiment, a test compound or a control compound is administered to the human subject, and the effect of a test compound on CAPI expression is determined by analyzing the expression of the CAPI or the mRNA encoding the same in a biological sample (*e.g.*, tissue, serum, plasma, or urine). A test compound that alters the expression of a CAPI can be identified by comparing the level of the CAPI or mRNA encoding the same in a subject or group of subjects treated with a control compound to that in a subject or group of subjects treated with a test compound. Alternatively, alterations in the expression of a CAPI can be identified by comparing the level of the CAPI or mRNA encoding the same in a subject or group of subjects before and after the administration of a test compound. Techniques known to those of skill in the art can be used to obtain the biological sample and analyze the mRNA or protein expression. For example, the Preferred Technology described herein can be used to assess changes in the level of a CAPI.

In another embodiment, test compounds that modulate the activity of a CAPI (or plurality of CAPIs) are identified in human subjects having chronic asthma, preferably those having mild to severe chronic asthma and most preferably those with mild chronic asthma. In this embodiment, a test compound or a control compound is administered to the human subject, and the effect of a test compound on the activity of a CAPI is determined. A test compound that alters the activity of a CAPI can be identified by comparing biological samples from subjects treated with a control compound to samples from subjects treated with the test compound. Alternatively, alterations in the activity of a CAPI can be identified by comparing the activity of a

CAPI in a subject or group of subjects before and after the administration of a test compound. The activity of the CAPI can be assessed by detecting in a biological sample (*e.g.*, tissue, serum, plasma, or urine) induction of a cellular signal transduction pathway of the CAPI (*e.g.*, intracellular Ca2+, diacylglycerol, IP3, etc.), catalytic or enzymatic activity of the CAPI or a binding partner thereof, or a cellular response, for example, cellular differentiation, or cell proliferation. Techniques known to those of skill in the art can be used to detect changes in the induction of a second messenger of a CAPI or changes in a cellular response. For example, RT-PCR can be used to detect changes in the induction of a cellular second messenger.

In a particular embodiment, a test compound that changes the level or expression of a CAPI towards levels detected in control subjects (*e.g.*, humans free from chronic asthma) is selected for further testing or therapeutic use. In another preferred embodiment, a test compound that changes the activity of a CAPI towards the activity found in control subjects (*e.g.*, humans free from chronic asthma) is selected for further testing or therapeutic use.

In another embodiment, test compounds that reduce the severity of one or more symptoms associated with chronic asthma are identified in human subjects having chronic asthma, preferably subjects having mild to severe chronic asthma and most preferably subjects with mild chronic asthma. In accordance with this embodiment, a test compound or a control compound is administered to the subjects, and the effect of a test compound on one or more symptoms of chronic asthma is determined. A test compound that reduces one or more symptoms can be identified by comparing the subjects treated with a control compound to the subjects treated with the test compound. Techniques known to physicians familiar with chronic asthma can be used to determine whether a test compound reduces one or more symptoms associated with chronic asthma.

In a preferred embodiment, a test compound that reduces the severity of one or more symptoms associated with chronic asthma in a human having chronic asthma is selected for further testing or therapeutic use.

## 5.17 Therapeutic and Prophylactic Compositions and Their Use

The invention provides methods of treatment (and prophylaxis) comprising administering to a subject an effective amount of a compound of the invention. In a preferred aspect, the compound is substantially purified (*e.g.*, substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human. In a specific embodiment, a non-human mammal is the subject.

Formulations and methods of administration that can be employed when the compound comprises a nucleic acid are described above; additional appropriate formulations and routes of administration are described below.

Various delivery systems are known and can be used to administer a compound of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction can be enteral or parenteral and include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment;

this may be achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, *e.g.*, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection into upper airways tissue. In another embodiment, the compound can be delivered in a vesicle, in particular a liposome (see Langer, 1990, Science 249:1527-1533; Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally ibid.)

In yet another embodiment, the compound can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, supra; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald et al., 1980, Surgery 88:507; Saudek et al., 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J., 1983, Macromol. Sci. Rev. Macromol. Chem. 23:61; see also Levy et al., 1985, Science 228:190; During et al., 1989, Ann. Neurol. 25:351; Howard et al., 1989, J. Neurosurg. 71:105 ). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the upper airways, thus requiring only a fraction of the systemic dose (see, *e.g.*, Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (1990, Science 249:1527-1533).

In another embodiment where the compound of the invention is a nucleic acid encoding a protein, the nucleic acid can be administered in vivo to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of

microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see *e.g.*, Joliot et al., 1991, Proc. Natl. Acad. Sci. USA 88:1864-1868), etc. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the subject. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the compound of the invention which will be effective in the treatment of chronic asthma can be determined by standard clinical techniques. In addition, in vitro assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each subject's circumstances. However, suitable dosage ranges for intravenous administration are generally about

20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects (a) approval by the agency of manufacture, use or sale for human administration, (b) directions for use, or both.

## 6 EXAMPLE 1: IDENTIFICATION OF PROTEINS DIFFERENTIALLY EXPRESSED IN THE TISSUE IN CHRONIC ASTHMA

Using the following procedure, proteins in primary cultures of human airway myofibroblasts were separated by isoelectric focusing followed by SDS-PAGE and analyzed. Different cultures were used, *infra*, and two different time points at 1 hour (referred to as "early") and 6 hours (referred to as "late") were taken. Parts 6.1.1 to 6.1.14 (inclusive) of the procedure set forth below are hereby designated as the "Reference Protocol".

### 6.1 MATERIALS AND METHODS

#### 6.1.1 Sample Preparation

Primary cultures of human airway myofibroblasts (Hall IP, Kotlikoff M, *Am J Physiol* 1995 Jan; 268(1 Pt 1): L1-11, Use of cultured airway myocytes for study of airway smooth muscle; Hirst SJ, *Eur Respir J* 1996 Apr; 9(4): 808-20, Airway smooth muscle cell culture: application to studies of airway wall remodelling and phenotype

plasticity in asthma.) were treated by one, or a successive combination of, the following:

- thrombin at 20 units/ml (Panettieri RA Jr, Hall IP, Maki CS, Murray RK, *Am J Respir Cell Mol Biol* 1995 Aug;13(2):205-16, Alpha-Thrombin increases cytosolic calcium and induces human airway smooth muscle cell proliferation).
- dexamethasone at 1 μM (glucocorticoid).
- theophylline 100 μM (Billington CK, Joseph SK, Swan C, Scott MG, Jobson TM, Hall IP, *Am J Physiol* 1999 Mar;276(3 Pt 1):L412-9, Modulation of human airway smooth muscle proliferation by type 3 phosphodiesterase inhibition).

The experimental design was as follows:

| | Experiment title | Sample conditions | Control conditions |
|---|---|---|---|
| A | early inflammatory asthmatic response | Stimulation with thrombin (20 units/ml) for 1 hour | Unstimulated subconfluent monolayer |
| D | late inflammatory asthmatic response | Stimulation with thrombin (20 units/ml) for 6 hours | Unstimulated subconfluent monolayer |
| B | early response to glucocorticoids in the presence of inflammation | Stimulation with thrombin (20 units/ml) for 1 hour with dexamethasone 1 μM for 2 hour | Stimulation with thrombin (20 units/ml) for 1 hour |
| E | late response to glucocorticoids in the presence of inflammation | Stimulation with thrombin (20 units/ml) for 6 hours with dexamethasone 1 μM for 7 hours | Stimulation with thrombin (20 units/ml) for 6 hours |
| C | early response to phosphodiesterase inhibitors in the presence of inflammation | Stimulation with thrombin (20 units/ml) for 1 hour with theophylline 100 μM for 2 hours | Stimulation with thrombin (20 units/ml) for 1 hour |
| F | late response to phosphodiesterase inhibitors in the presence of | Stimulation with thrombin (20 units/ml) for 6 hours with theophylline 100 μM for 7 | Stimulation with thrombin (20 units/ml) for 6 hours |

| | Experiment title | Sample conditions | Control conditions |
|---|---|---|---|
| | inflammation | hours | |

6.1.2 Isoelectric Focusing

Isoelectric focusing (IEF), was performed using the Immobiline7 DryStrip Kit (Pharmacia BioTech), following the procedure described in the manufacturer's instructions, see Instructions for Immobiline7 DryStrip Kit, Pharmacia, # 18-1038-63, Edition AB (incorporated herein by reference in its entirety). Immobilized pH Gradient (IPG) strips (18cm, pH 3-10 non-linear strips; Pharmacia Cat. # 17-1235-01) were rehydrated overnight at 20°C in a solution of 8M urea, 2% (w/v) CHAPS, 10mM DTT, 2% (v/v) Resolytes 3.5-10, as described in the Immobiline DryStrip Users Manual. For IEF, 50ml of supernatant (prepared as above) was loaded onto a strip, with the cup-loading units being placed at the basic end of the strip. The loaded gels were then covered with mineral oil (Pharmacia 17-3335-01) and a voltage was immediately applied to the strips according to the following profile, using a Pharmacia EPS3500XL power supply (Cat 19-3500-01):

Initial voltage = 300V for 2 hrs
Linear Ramp from 300V to 3500V over 3hrs
Hold at 3500V for 19hrs

For all stages of the process, the current limit was set to 10mA for 12 gels, and the wattage limit to 5W. The temperature was held at 20°C throughout the run.

6.1.3 Gel Equilibration and SDS-PAGE

After the final 19hr step, the strips were immediately removed and immersed for 10 mins at 20°C in a first solution of the following composition: 6M urea; 2% (w/v) DTT; 2% (w/v) SDS; 30% (v/v) glycerol (Fluka 49767); 0.05M Tris/HCl, pH 6.8 (Sigma Cat T-1503). The strips were removed from the first solution and immersed for 10 mins at 20°C in a second solution of the following composition: 6M urea; 2% (w/v) iodoacetamide (Sigma I-6125); 2% (w/v) SDS; 30% (v/v) glycerol;

0.05M Tris/HCl, pH 6.8. After removal from the second solution, the strips were loaded onto supported gels for SDS-PAGE according to Hochstrasser et al., 1988, Analytical Biochemistry 173: 412-423 (incorporated herein by reference in its entirety), with modifications as specified below.

6.1.4 Preparation of supported gels

The gels were cast between two glass plates of the following dimensions: 23cm wide x 24cm long (back plate); 23cm wide x 24cm long with a 2cm deep notch in the central 19cm (front plate). To promote covalent attachment of SDS-PAGE gels, the back plate was treated with a 0.4% solution of g-methacryl-oxypropyltrimethoxysilane in ethanol (BindSilaneJ; Pharmacia Cat. # 17-1330-01). The front plate was treated with (RepelSilaneJ Pharmacia Cat. # 17-1332-01) to reduce adhesion of the gel. Excess reagent was removed by washing with water, and the plates were allowed to dry. At this stage, both as identification for the gel, and as a marker to identify the coated face of the plate, an adhesive bar-code was attached to the back plate in a position such that it would not come into contact with the gel matrix.

The dried plates were assembled into a casting box with a capacity of 13 gel sandwiches. The top and bottom plates of each sandwich were spaced by means of 1mm thick spacers, 2.5 cm wide. The sandwiches were interleaved with acetate sheets to facilitate separation of the sandwiches after gel polymerization. Casting was then carried out according to Hochstrasser et al., op. cit.

A 9-16% linear polyacrylamide gradient was cast, extending up to a point 2cm below the level of the notch in the front plate, using the Angelique gradient casting system (Large Scale Biology). Stock solutions were as follows. Acrylamide (40% in water) was from Serva (Cat. # 10677). The cross-linking agent was PDA (BioRad 161-0202), at a concentration of 2.6% (w/w) of the total starting monomer content. The gel buffer was 0.375M Tris/HCl, pH 8.8. The polymerization catalyst was 0.05% (v/v) TEMED (BioRad 161-0801), and the initiator was 0.1% (w/v) APS (BioRad 161-0700). No SDS was included in the gel and no stacking gel was used.

The cast gels were allowed to polymerize at 20°C overnight, and then stored at 4°C in sealed polyethylene bags with 6ml of gel buffer, and were used within 4 weeks.

6.1.5 SDS-PAGE

A solution of 0.5% (w/v) agarose (Fluka Cat 05075) was prepared in running buffer (0.025M Tris, 0.198M glycine (Fluka 50050), 1% (w/v) SDS, supplemented by a trace of bromophenol blue). The agarose suspension was heated to 70°C with stirring, until the agarose had dissolved. The top of the supported 2nd D gel was filled with the agarose solution, and the equilibrated strip was placed into the agarose, and tapped gently with a palette knife until the gel was intimately in contact with the 2nd D gel. The gels were placed in the 2nd D running tank, as described by Amess et al., 1995, Electrophoresis 16: 1255-1267 (incorporated herein by reference in its entirety). The tank was filled with running buffer (as above) until the level of the buffer was just higher than the top of the region of the 2nd D gels which contained polyacrylamide, so as to achieve efficient cooling of the active gel area. Running buffer was added to the top buffer compartments formed by the gels, and then voltage was applied immediately to the gels using a Consort E-833 power supply. For 1 hour, the gels were run at 20mA/gel. The wattage limit was set to 150W for a tank containing 6 gels, and the voltage limit was set to 600V. After 1 hour, the gels were then run at 40mA/gel, with the same voltage and wattage limits as before, until the bromophenol blue line was 0.5cm from the bottom of the gel. The temperature of the buffer was held at 16°C throughout the run. Gels were not run in duplicate.

6.1.6 Staining

Upon completion of the electrophoresis run, the gels were immediately removed from the tank for fixation. The top plate of the gel cassette was carefully removed, leaving the gel bonded to the bottom plate. The bottom plate with its attached gel was then placed into a staining apparatus, which can accommodate 12 gels. The gels were completely immersed in fixative solution of 40% (v/v) ethanol

(BDH 28719), 10% (v/v) acetic acid (BDH 100016X), 50% (v/v) water (MilliQ-Millipore), which was continuously circulated over the gels. After an overnight incubation, the fixative was drained from the tank, and the gels were primed by immersion in 7.5% (v/v) acetic acid, 0.05% (w/v) SDS, 92.5% (v/v) water for 30 mins. The priming solution was then drained, and the gels were stained by complete immersion for 4 hours in a staining solution of Pyridinium, 4-[2-[4-(dipentylamino)-2-trifluoromethylphenyl] ethenyl]-1-(sulfobutyl)-, inner salt, prepared by diluting a stock solution of this dye (2mg/ml in DMSO) in 7.5% (v/v) aqueous acetic acid to give a final concentration of 1.2 mg/l; the staining solution was vacuum filtered through a 0.4µm filter (Duropore) before use.

6.1.7 Imaging of the gel

A computer-readable output was produced by imaging the fluorescently stained gels with the Apollo 2 scanner (Oxford Glycosciences, Oxford, UK) described in section 5.2, supra. This scanner has a gel carrier with four integral fluorescent markers (Designated M1, M2, M3, M4) that are used to correct the image geometry and are a quality control feature to confirm that the scanning has been performed correctly.

For scanning, the gels were removed from the stain, rinsed with water and allowed to air dry briefly, and imaged on the Apollo 2 scanner. After imaging, the gels were sealed in polyethylene bags containing a small volume of staining solution, and then stored at 4°C.

6.1.8 Digital Analysis of the Data

The data were processed as described in U.S. Patent No. 6,064,754 at Sections 5.4 and 5.5 (incorporated herein by reference), as set forth more particularly below.

The output from the scanner was first processed using the MELANIE7 II 2D PAGE analysis program (Release 2.2, 1997, BioRad Laboratories, Hercules, California, Cat. # 170-7566) to autodetect the registration points, M1, M2, M3 and

M4; to autocrop the images (*i.e.*, to eliminate signals originating from areas of the scanned image lying outside the boundaries of the gel, *e.g.* the reference frame); to filter out artifacts due to dust; to detect and quantify features; and to create image files in GIF format. Features were detected using the following parameters:

Smooths =2

Laplacian threshold 50

Partials threshold 1

Saturation = 100

Peakedness = 0

Minimum Perimeter = 10

6.1.9 Assignment of pI and MW Values

Landmark identification was used to determine the pI and MW of features detected in the images. Fifteen landmark features, designated MF1 to MF15, were identified in a standard tissue image. These landmark features are identified in Figure 2 and were assigned the pI and/or MW values identified in Table VIII.

Table VIII. Landmark Features Used In This Study

Table VIII

| MF# | pI | MW (D) |
|---|---|---|
| MF1 | 6.07 | 144276 |
| MF2 | 4.78 | 116528 |
| MF3 | 5.85 | 104386 |
| MF4 | 5.16 | 80625 |
| MF5 | 5.36 | 71346 |
| MF6 | 6.72 | 62494 |
| MF7 | 4.49 | |
| MF8 | 5.72 | 57516 |
| MF9 | 7.53 | 49018 |

| | | |
|---|---|---|
| MF10 | 8.8 | 39282 |
| MF11 | 5.49 | 35350 |
| MF12 | | 27823 |
| MF13 | 6.18 | 23764 |
| MF14 | 8.27 | 14080 |
| MF15 | 7.69 | 11240 |

As many of these landmarks as possible were identified in each gel image of the dataset. Each feature in the study gels was then assigned a pI value by linear interpolation or extrapolation (using the MELANIE7-II software) to the two nearest landmarks, and was assigned a MW value by linear interpolation or extrapolation (using the MELANIE7-II software) to the two nearest landmarks.

6.1.10 Matching With Primary Master Image

Images were edited to remove gross artifacts such as dust, to reject images which had gross abnormalities such as smearing of protein features, or were of too low a loading or overall image intensity to allow identification of more than the most intense features, or were of too poor a resolution to allow accurate detection of features. Images were then compared by pairing with one common image from the whole sample set. This common image, the "primary master image", was selected on the basis of protein load (maximum load consistent with maximum feature detection), a well resolved myoglobin region, (myoglobin was used as an internal standard), and general image quality. Additionally, the primary master image was chosen to be an image which appeared to be generally representative of all those to be included in the analysis. (This process by which a primary master gel was judged to be representative of the study gels was rechecked by the method described below and in the event that the primary master gel was seen to be unrepresentative, it was rejected and the process repeated until a representative primary master gel was found.)

Each of the remaining study gel images was individually matched to the primary master image such that common protein features were paired between the primary master image and each individual study gel image as described below.

6.1.11 Cross-matching Between Samples

To facilitate statistical analysis of large numbers of samples for purposes of identifying features that are differentially expressed, the geometry of each study gel was adjusted for maximum alignment between its pattern of protein features, and that of the primary master, as follows. Each of the study gel images was individually transformed into the geometry of the primary master image using a multi-resolution warping procedure. This procedure corrects the image geometry for the distortions brought about by small changes in the physical parameters of the electrophoresis separation process from one sample to another. The observed changes are such that the distortions found are not simple geometric distortions, but rather a smooth flow, with variations at both local and global scale.

The fundamental principle in multi-resolution modeling is that smooth signals may be modeled as an evolution through 'scale space', in which details at successively finer scales are added to a low resolution approximation to obtain the high resolution signal. This type of model is applied to the flow field of vectors (defined at each pixel position on the reference image) and allows flows of arbitrary smoothness to be modeled with relatively few degrees of freedom. Each image is first reduced to a stack, or pyramid, of images derived from the initial image, but smoothed and reduced in resolution by a factor of 2 in each direction at every level (Gaussian pyramid) and a corresponding difference image is also computed at each level, representing the difference between the smoothed image and its progenitor (Laplacian pyramid). Thus the Laplacian images represent the details in the image at different scales.

To estimate the distortion between any 2 given images, a calculation was performed at level 7 in the pyramid (*i.e.* after 7 successive reductions in resolution). The Laplacian images were segmented into a grid of 16x16 pixels, with 50% overlap between adjacent grid positions in both directions, and the cross correlation between

corresponding grid squares on the reference and the test images was computed. The distortion displacement was then given by the location of the maximum in the correlation matrix. After all displacements had been calculated at a particular level, they were interpolated to the next level in the pyramid, applied to the test image, and then further corrections to the displacements were calculated at the next scale.

The warping process brought about good alignment between the common features in the primary master image, and the images for the other samples. The MELANIE7 II 2D PAGE analysis program was used to calculate and record approximately 500-700 matched feature pairs between the primary master and each of the other images. The accuracy of this program was significantly enhanced by the alignment of the images in the manner described above. To improve accuracy still further, all pairings were finally examined by eye in the MelView interactive editing program and residual recognizably incorrect pairings were removed. Where the number of such recognizably incorrect pairings exceeded the overall reproducibility of the Preferred Technology (as measured by repeat analysis of the same biological sample) the gel selected to be the primary master gel was judged to be insufficiently representative of the study gels to serve as a primary master gel. In that case, the gel chosen as the primary master gel was rejected, and different gel was selected as the primary master gel, and the process was repeated.

All the images were then added together to create a composite master image, and the positions and shapes of all the gel features of all the component images were super-imposed onto this composite master as described below.

Once all the initial pairs had been computed, corrected and saved, a second pass was performed whereby the original (unwarped) images were transformed a second time to the geometry of the primary master, this time using a flow field computed by smooth interpolation of the multiple tie-points defined by the centroids of the paired gel features. A composite master image was thus generated by initial*izing* the primary master image with its feature descriptors. As each image was transformed into the primary master geometry, it was digitally summed pixel by pixel into the composite master image, and the features that had not been paired by the

procedure outlined above were likewise added to the composite master image description, with their centroids adjusted to the master geometry using the flow field correction.

The final stage of processing was applied to the composite master image and its feature descriptors, which now represent all the features from all the images in the study transformed to a common geometry. The features were grouped together into linked sets or "clusters", according to the degree of overlap between them. Each cluster was then given a unique identifying index, the molecular cluster index (MCI).

An MCI identifies a set of matched features on different images. Thus an MCI represents a protein or proteins eluting at equivalent positions in the 2D separation in different samples.

6.1.12 Construction of Profiles

After matching all component gels in the study to the final composite master image, the intensity of each feature was measured and stored. The end result of this analysis was the generation of a digital profile which contained, for each identified feature: 1) a unique identification code relative to corresponding feature within the composite master image (MCI), 2) the x, y coordinates of the features within the gel, 3) the isoelectric point (pI) of the CAFs, 4) the apparent molecular weight (MW) of the CAFs, 5) the signal value, 6) the standard deviation for each of the preceding measurements, and 7) a method of linking the MCI of each feature to the master gel to which this feature was matched. By virtue of a Laboratory Information Management System (LIMS), this MCI profile was traceable to the actual stored gel from which it was generated, so that proteins identified by computer analysis of gel profile databases could be retrieved. The LIMS also permitted the profile to be traced back to an original sample or patient.

6.1.13 Statistical Analysis of the Profiles

Using the protein expression database, matching protein features present in test primary cultures and in control primary cultures were compared. The protein

features were present in at least 68% of the sample group; with expression profiles exceeding a significant fold change threshold (in this case, a threshold of 1.7 was used) were identified as Chronic Asthma Associated Features (CAFs).

6.1.14 Recovery and analysis of selected proteins

Proteins in CAFs were robotically excised and processed to generate tryptic digest peptides. Tryptic peptides were analyzed by mass spectrometry using a PerSeptive Biosystems Voyager- DETM STR Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometer, and selected tryptic peptides were analyzed by tandem mass spectrometry (MS/MS) using a Micromass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Micromass, Altrincham, U.K.) equipped with a nanoflowTM electrospray Z-spray source. For partial amino acid sequencing and identification of CAPIs uninterpreted tandem mass spectra of tryptic peptides were searched using the SEQUEST search program (Eng et al., 1994, J. Am. Soc. Mass Spectrom. 5:976-989), version v.C.1. Criteria for database identification included: the cleavage specificity of trypsin; the detection of a suite of a, b and y ions in peptides returned from the database, and a mass increment for all Cys residues to account for carbamidomethylation. The database searched was database constructed of protein entries in the non-redundant database held by the National Centre for Biotechnology Information (NCBI), which is accessible at http://www.ncbi.nlm.nih.gov/. Following identification of proteins through spectral-spectral correlation using the SEQUEST program, masses detected in MALDI-TOF mass spectra were assigned to tryptic digest peptides within the proteins identified. In cases where no proteins could be identified through searching with uninterpreted MS/MS spectra of tryptic digest peptides using the SEQUEST program; tandem mass spectra of the peptides were interpreted manually, using methods known in the art. (In the case of interpretation of low-energy fragmentation mass spectra of peptide ions see Gaskell et al., 1992, Rapid Commun. Mass Spectrom. 6:658-662). The method described in PCT Application No. PCT/GB01/04034, which is incorporated herein by reference in its entirety, was also used to interpret mass spectra.

Alternatively sequences were identified using peptide mass data derived from mass spectrometer analysis and then used for a MOWSE database search. Peptide mass information can provide a 'fingerprint' signature sufficiently discriminating to allow for the unique and rapid identification of unknown sample proteins, independent of other analytical methods such as protein sequence analysis. Practical experience has shown that sample proteins can be uniquely identified using as few as 3- 4 experimentally determined peptide masses when screened against a fragment database derived from over 50,000 proteins (D.J.C. Pappin, P. Hojrup and A.J. Bleasby 'Rapid Identification of Proteins by Peptide-Mass Fingerprinting'. Current Biology (1993), vol 3, 327-332. and http://www.hgmp.mrc.ac.uk/Bioinformatics/Webapp/mowse/).

## 6.2 RESULTS

These initial experiments identified 18 features that were differentially present in a least one of the experimental conditions described above. Details of these CAFs are provided in Table I and in the tables below.

Table IX: Increased in an early asthmatic response

**A** = Fold change following 1h thrombin, relative to untreated cells

**B** = Fold change following 1h thrombin and 1h Dexamethasone relative to cells treated with thrombin alone.

**C** = Fold change following 1 h thrombin and 1h theophylline relative to cells treated with thrombin alone.

| CAF | pI | MW | A | B | C |
|---|---|---|---|---|---|
| CAF-2 | 5.16 | 119778 | 7.832 | | 4.912 |
| CAF-22 | 5.27 | 26368 | 1.733 | -2.909 | -2.096 |
| CAF-29 | 7.46 | 60270 | 2.597 | | |
| CAF-34 | 6.47 | 33980 | 2.741 | | |

Table X: Decreased in an early asthmatic response

**A** = Fold change following 1h thrombin, relative to untreated cells

**B** = Fold change following 1h thrombin and 1h Dexamethasone relative to cells treated with thrombin alone.

**C** = Fold change following 1 h thrombin and 1h theophylline relative to cells treated with thrombin alone.

| CAF# | pI | MW | A | B | C |
|---|---|---|---|---|---|
| CAF-1 | 5.37 | 122123 | -4.393 | 4.713 | 3.518 |
| CAF-3 | 5.3 | 116172 | -2.976 | 2.438 | |
| CAF-5 | 5.02 | 108632 | -3.486 | | 2.306 |
| CAF-12 | 6.55 | 61580 | -51.041 | 21.529 | 39.410 |
| CAF-13 | 5.62 | 60360 | -2.425 | 5.677 | 4.288 |
| CAF-15 | 5.28 | 55571 | -14.045 | 4.619 | 16.824 |
| CAF-18 | 7.67 | 35754 | -19.780 | 38.553 | 26.692 |
| CAF-20 | 5.07 | 30903 | -16.512 | 20.554 | 29.086 |
| CAF-21 | 7.68 | 28655 | -4.110 | | |
| CAF-24 | 5.23 | 49444 | -332.863 | 75.754 | 19.892 |
| CAF-25 | 5.62 | 37110 | -8.122 | 5.245 | 10.216 |

Table XI: Increased in a late asthmatic response

**D** = Fold change following 6h thrombin, relative to untreated cells

**E** = Fold change following 6h thrombin and 7h Dexamethasone relative to cells treated with thrombin alone.

**F** = Fold change following 6 h thrombin and 7h theophylline relative to cells treated with thrombin alone.

| CAF# | pI | MW | D | E | F |
|---|---|---|---|---|---|
| CAF-1 | 5.37 | 122123 | 2.463 | | -2.122 |
| CAF-2 | 5.16 | 119778 | 14.593 | | |
| CAF-3 | 5.3 | 116172 | 2.120 | | |
| CAF-13 | 5.62 | 60360 | 3.292 | | |
| CAF-15 | 5.28 | 55571 | 3.037 | -2.423 | -2.077 |

| CAF # | pI | MW | D | E | F |
|---|---|---|---|---|---|
| CAF-18 | 7.67 | 35754 | 16.517 | -15.966 | 2.839 |
| CAF-21 | 7.68 | 28655 | 4.186 | | |
| CAF-29 | 7.46 | 60270 | 2.169 | -2.142 | |
| CAF-34 | 6.47 | 33980 | 5.005 | | |

Table XII: Decreased in a late asthmatic response

**D** = Fold change following 6h thrombin, relative to untreated cells

**E** = Fold change following 6h thrombin and 6h Dexamethasone relative to cells treated with thrombin alone.

**F** = Fold change following 6 h thrombin and 6h theophylline relative to cells treated with thrombin alone.

| CAF # | pI | MW | D | E | F |
|---|---|---|---|---|---|
| CAF-5 | 5.02 | 108632 | -2.846 | | |
| CAF-19 | 7.91 | 34254 | -2.815 | | 2.800 |
| CAF-25 | 5.62 | 37110 | -4.750 | | 19.320 |
| CAF-33 | 4.88 | 40521 | -4.459 | | 2.031 |
| CAF-35 | 8.16 | 22061 | -3.705 | | 2.182 |

Partial amino acid sequences were determined for the differentially present CAPIs in these CAFs. Details of these CAPIs are provided in Tables III and IV.

The present invention is not to be limited in terms of the particular embodiments described in this application, which are intended as single illustrations of individual aspects of the invention. Functionally equivalent methods and apparatus within the scope of the invention, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications and variations are intended to fall within the scope of the appended claims. The contents of each reference, patent and patent application cited in this application is hereby incorporated by reference in its entirety.

When a reference is made herein to a method of treating or preventing a disease or condition using a particular agent or combination of agents, it is to be understood that such a reference is intended to include the use of that agent or combination of agents in the preparation of a medicament for the treatment or prevention of the disease or condition.

Preferred features of each aspect of the invention are as for each of the other aspects *mutatis mutandis*.

# CLAIMS

1. A method for screening, diagnosis or prognosis of chronic asthma in a subject, for determining the stage or severity of chronic asthma in a subject, for identifying a subject at risk of developing chronic asthma, or for monitoring the effect of therapy administered to a subject having chronic asthma, said method comprising:

a) analyzing a test sample of tissue from the subject by two dimensional electrophoresis to generate a two-dimensional array of features, said array comprising at least one chosen feature whose relative abundance correlates with the presence, absence, stage or severity of chronic asthma or predicts the onset or course of chronic asthma; and

b) comparing the abundance of each chosen feature in the test sample with the abundance of that chosen feature in a sample from one or more persons free from chronic asthma, or with a previously determined reference range for that feature in subjects free from chronic asthma, or with the abundance at least one Expression Reference Feature (ERF) in the test sample.

2. The method of claim 1, wherein the test sample is taken from the upper airways.

3. The method of claim 1 or claim 2, wherein said method is for screening or diagnosis of chronic asthma and the relative abundance of at least one chosen feature correlates with the presence or absence of chronic asthma.

4. The method of claim 1 or claim 2, wherein said method is for monitoring the effect of therapy administered to a subject having chronic asthma and the relative abundance of at least one chosen feature correlates with the severity of chronic asthma.

5. The method of any one of the preceding claims, wherein step (b) comprises comparing the abundance of each chosen feature in the sample with the abundance of

that chosen feature in tissue from one or more persons free from chronic asthma or with a previously determined reference range for that chosen feature in subjects free from chronic asthma.

6. The method of any one of the preceding claims, wherein step (b) comprises quantitatively detecting one or more of the following Chronic Asthma-Associated Features (CAFs): CAF-1, CAF-2, CAF-3, CAF-5, CAF-12, CAF-13, CAF-15, CAF-18, CAF-19, CAF-20, CAF-21, CAF-22, CAF-24, CAF-25, CAF-29, CAF-33, CAF-34, CAF-35.

7. The method according to any one of the preceding claims wherein step (a) comprises isoelectric focusing followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

8. A method for screening, diagnosis or prognosis of chronic asthma in a subject, for determining the stage or severity of chronic asthma in a subject, for identifying a subject at risk of developing chronic asthma, or for monitoring the effect of therapy administered to a subject having chronic asthma, said method comprising quantitatively detecting, in a sample of tissue from the subject, at least one of the following Chronic Asthma-Associated Protein Isoforms (CAPIs): CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38.

9. The method according to claim 8, wherein the step of quantitatively detecting comprises testing at least one aliquot of the sample, said step of testing comprising:

1) contacting the aliquot with an antibody that is immunospecific for a preselected CAPI; and

2) quantitatively measuring any binding that has occurred between the antibody and at least one species in the aliquot.

10. The method according to claim 9, wherein the step of quantitatively detecting comprises testing a plurality of aliquots with a plurality of antibodies for quantitative detection of a plurality of preselected CAPIs.

11. The method according to claim 9 or 10, wherein the or each antibody is a monoclonal antibody.

12. A preparation comprising one of the following isolated chronic asthma-Associated Protein Isoforms (CAPIs): CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38.

13. A preparation of claim 12, wherein the or each CAPI is/are isolated and/or recombinant.

14. A kit comprising the preparation of claims 12 or 13.

15. A kit comprising a plurality of distinct preparations of claim 14.

16. An antibody capable of immunospecific binding to one of the following chronic asthma-Associated Protein Isoforms (CAPIs): CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38.

17. The antibody of claim 16, which is a monoclonal antibody.

18. The antibody of claim 16 or 17, which binds to the CAPI with greater affinity than to another variant of the CAPI.

19. The antibody of claim 16 or 17, which binds to the CAPI with greater affinity than to any other variant of the CAPI.

20. A kit comprising the antibody of any one of claims 16 to 19.

21. A kit comprising a plurality of distinct antibodies of any one of claims 16 to 19.

22. A pharmaceutical composition comprising a therapeutically effective amount of an antibody of any one of claims 16 to 19 and a pharmaceutically acceptable carrier.

23. A method of treating or preventing chronic asthma comprising administering to a subject in need of such treatment or prevention a therapeutically effective amount of one of the following chronic Asthma Associated Protein Isoforms (CAPIs): CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38.

24. A method of treating or preventing chronic asthma comprising administering to a subject in need of such treatment or prevention a therapeutically effective amount of an antibody as claimed in any of claims 16 to 19.

25. A method of treating or preventing chronic asthma comprising administering to a subject in need of such treatment or prevention a therapeutically effective amount of a nucleic acid encoding one of the following chronic asthma-Associated Protein Isoforms (CAPIs): CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38.

26. A method of treating or preventing chronic asthma comprising administering to a subject in need of such treatment or prevention a therapeutically effective amount of a nucleic acid that inhibits the function of one or more of the following chronic asthma-Associated Protein Isoforms (CAPIs): CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38.

27. The method of claim 26, wherein the nucleic acid is a CAPI antisense nucleic acid or ribozyme.

28. A method of screening for agents that interact with a CAPI, a CAPI fragment, a CAPI-related polypeptide or a CAPI fusion protein said method comprising:

1) contacting a CAPI, a CAPI fragment, a CAPI-related polypeptide or a CAPI fusion protein with a candidate agent; and

2) determining whether or not the candidate agent interacts with the CAPI, the CAPI fragment, the CAPI-related polypeptide or the CAPI fusion protein.

29. The method of claim 28, wherein the CAPI, the CAPI fragment, the CAPI-related polypeptide or the CAPI fusion protein is expressed by cells.

30. The method of claim 29, wherein the cells express a recombinant CAPI, a recombinant CAPI fragment, a recombinant CAPI-related polypeptide or a CAPI fusion protein.

31. A method of screening for agents that modulate the expression or activity of a CAPI, a CAPI fragment, a CAPI-related polypeptide or a CAPI fusion protein comprising:

1) contacting a first population of cells expressing a CAPI, a CAPI fragment, a CAPI-related polypeptide or a CAPI fusion protein with a candidate agent;

2) contacting a second population of cells expressing said CAPI, said CAPI fragment, said CAPI-related polypeptide or said CAPI fusion protein with a control agent; and

3) comparing the level of said CAPI, said CAPI fragment, said CAPI-related polypeptide or said CAPI fusion protein or mRNA encoding said CAPI, said CAPI fragment, said CAPI-related polypeptide or said CAPI fusion protein in the first and second populations of cells, or comparing the level of induction of a cellular second messenger in the first and second populations of cells.

32. The method of claim 31, wherein the level of said CAPI, said CAPI fragment, said CAPI-related polypeptide or said CAPI fusion protein, mRNA encoding said CAPI, said CAPI fragment, said CAPI-related polypeptide, said CAPI fusion protein or said cellular second messenger is greater in the first population of cells than in the second population of cells.

33. The method of claim 31, wherein the level of said CAPI, said CAPI fragment, said CAPI-related polypeptide or said CAPI fusion protein, mRNA encoding said CAPI, said CAPI fragment, said CAPI-related polypeptide, said CAPI fusion protein or said cellular second messenger is less in the first population of cells than in the second population of cells.

34. A method of screening for or identifying agents that modulate the expression or activity of a CAPI, a CAPI fragment, a CAPI-related polypeptide or a CAPI fusion protein comprising:

1) administering a candidate agent to a first mammal or group of mammals;

2) administering a control agent to a second mammal or group of mammals; and

3) comparing the level of expression of the CAPI, the CAPI fragment, the CAPI-related polypeptide, or the CAPI fusion protein or of mRNA encoding the CAPI, the CAPI fragment, the CAPI-related polypeptide, or the CAPI fusion protein in the first and second groups, or comparing the level of induction of a cellular second messenger in the first and second groups.

35. The method of claim 34, wherein the mammals are animal models for chronic asthma.

36. The method of claim 34 or 35, wherein the level of expression of said CAPI, said CAPI fragment, said CAPI-related polypeptide or said CAPI fusion protein, mRNA encoding said CAPI, said CAPI fragment, said CAPI-related polypeptide or said CAPI fusion protein, or of said cellular second messenger is greater in the first group than in the second group.

37. The method of claim 34 or 35, wherein the level of expression of said CAPI, said CAPI fragment, said CAPI-related polypeptide, or said CAPI fusion protein, mRNA encoding said CAPI, said CAPI fragment, said CAPI-related polypeptide, or said CAPI fusion protein, or of said cellular second messenger is less than in the first group than in the second group.

38. The method of any one of claims 34 to 37, wherein the levels of said CAPI, said CAPI fragment, said CAPI-related polypeptide, or said CAPI fusion protein, mRNA encoding said CAPI, said CAPI fragment, said CAPI-related polypeptide, or said CAPI fusion protein, or of said cellular second messenger in the first and second groups are further compared to the level of said CAPI, said CAPI fragment, said CAPI-related polypeptide, or said CAPI fusion protein or said mRNA encoding said

CAPI, said CAPI fragment, said CAPI-related polypeptide, or said CAPI fusion protein in normal control mammals.

39. The method of any one of claims 34 to 38, wherein administration of said candidate agent modulates the level of said CAPI, said CAPI fragment, said CAPI-related polypeptide or said CAPI fusion protein, or said mRNA encoding said CAPI, said CAPI fragment, said CAPI-related polypeptide, or said CAPI fusion protein, or said cellular second messenger in the first group towards the levels of said CAPI, said CAPI fragment, said CAPI-related polypeptide or said CAPI fusion protein or said mRNA or said cellular second messenger in the second group.

40. The method of any one of claims 34 to 39, wherein said mammals are human subjects having chronic asthma.

41. A method of screening for or identifying agents that interact with a CAPI, a CAPI fragment, a CAPI-related polypeptide or a CAPI fusion protein, comprising

a) contacting a candidate agent with the CAPI, the CAPI fragment, the CAPI-related polypeptide, or the CAPI fusion protein, and

b) quantitatively detecting binding, if any, between the agent and the CAPI, the CAPI fragment, the CAPI-related polypeptide, or the CAPI fusion protein.

42. A method of screening for or identifying agents that modulate the activity of a CAPI, a CAPI fragment, a CAPI-related polypeptide, or a CAPI fusion protein comprising

a) in a first aliquot, contacting a candidate agent with the CAPI, the CAPI fragment, the CAPI-related polypeptide or the CAPI fusion protein, and

b) comparing the activity of the CAPI, the CAPI fragment, the CAPI-related polypeptide or the CAPI fusion protein in the first aliquot after addition of the candidate agent with the activity of the CAPI, the CAPI fragment, the

CAPI-related polypeptide or the CAPI fusion protein in a control aliquot, or with a previously determined reference range.

43. The method according to claim 41 or 42, wherein the CAPI, the CAPI fragment, the CAPI-related polypeptide or the CAPI fusion protein is a recombinant protein.

44. The method according to claim 41 or 42, wherein the CAPI, the CAPI fragment, the CAPI-related polypeptide or the CAPI fusion protein is immobilized on a solid phase.

45. A method for screening, diagnosis or prognosis of chronic asthma in a subject or for monitoring the effect of an anti-chronic asthma drug or therapy administered to a subject, comprising:

a) contacting at least one oligonucleotide probe comprising 10 or more consecutive nucleotides complementary to a nucleotide sequence encoding a CAPI chosen from CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38 with an RNA obtained from a biological sample from the subject or with cDNA copied from the RNA wherein said contacting occurs under conditions that permit hybridization of the probe to the nucleotide sequence if present;

b) detecting hybridization, if any, between the probe and the nucleotide sequence; and

c) comparing the hybridization, if any, detected in step (b) with the hybridization detected in a control sample, or with a previously determined reference range.

46. The method of claim 45, wherein step (a) comprises contacting a plurality of oligonucleotide probes comprising 10 or more consecutive nucleotides complementary

to a nucleotide sequence encoding a CAPI chosen from CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38 with an RNA obtained from a biological sample from the subject or with cDNA copied from the RNA wherein said contacting occurs under conditions that permit hybridization of the probe to the nucleotide sequence if present.

47. The method of claim 45, wherein step (a) includes the step of hybridizing the nucleotide sequence to a DNA array, wherein one or more members of the array are the probes complementary to a plurality of nucleotide sequences encoding distinct CAPIs.

48. A method of modulating the activity of one or more of the following Chronic Asthma-Associated Protein Isoforms (CAPIs): CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38, comprising the step of contacting a cell with an agent which specifically binds to one or more of the following Chronic Asthma-Associated Protein Isoforms (CAPIs): CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38, whereby the activity of the respective CAPI is modulated.

49. An agent that modulates the activity of one or more of the following Chronic Asthma-Associated Protein Isoforms (CAPIs): CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38, wherein said agent is identified by the method of any of claims 28-44.

50. An agent as claimed in claim 49 for use in the manufacture of a medicament for the treatment or prevention of chronic asthma.

51. A pharmaceutical composition, comprising: the agent of claims 49 or 50 and a pharmaceutically acceptable carrier.

52. A method of treating or preventing chronic asthma comprising administering to a subject in need of such treatment or prevention a therapeutically effective dose of an agent that modulates the activity of one or more of the following Chronic Asthma-Associated Protein Isoforms (CAPIs): CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38, whereby the symptoms of chronic asthma are ameliorated.

53. A method for identifying targets for therapeutic modulation of chronic asthma wherein the activity of one or more of the following Chronic Asthma-Associated Protein Isoforms (CAPIs): CAPI-1, CAPI-2, CAPI-3, CAPI-5, CAPI-12, CAPI-13, CAPI-15, CAPI-18, CAPI-19, CAPI-20, CAPI-21, CAPI-22, CAPI-24, CAPI-25, CAPI-29, CAPI-30, CAPI-34, CAPI-35, CAPI-36, CAPI-37, CAPI-38 is utilized as a measure to determine whether a candidate target is effective for modulation of chronic asthma.

**Figure 1**

**Characterization of a Feature and relationship of a Feature and Protein Isoform(s)**

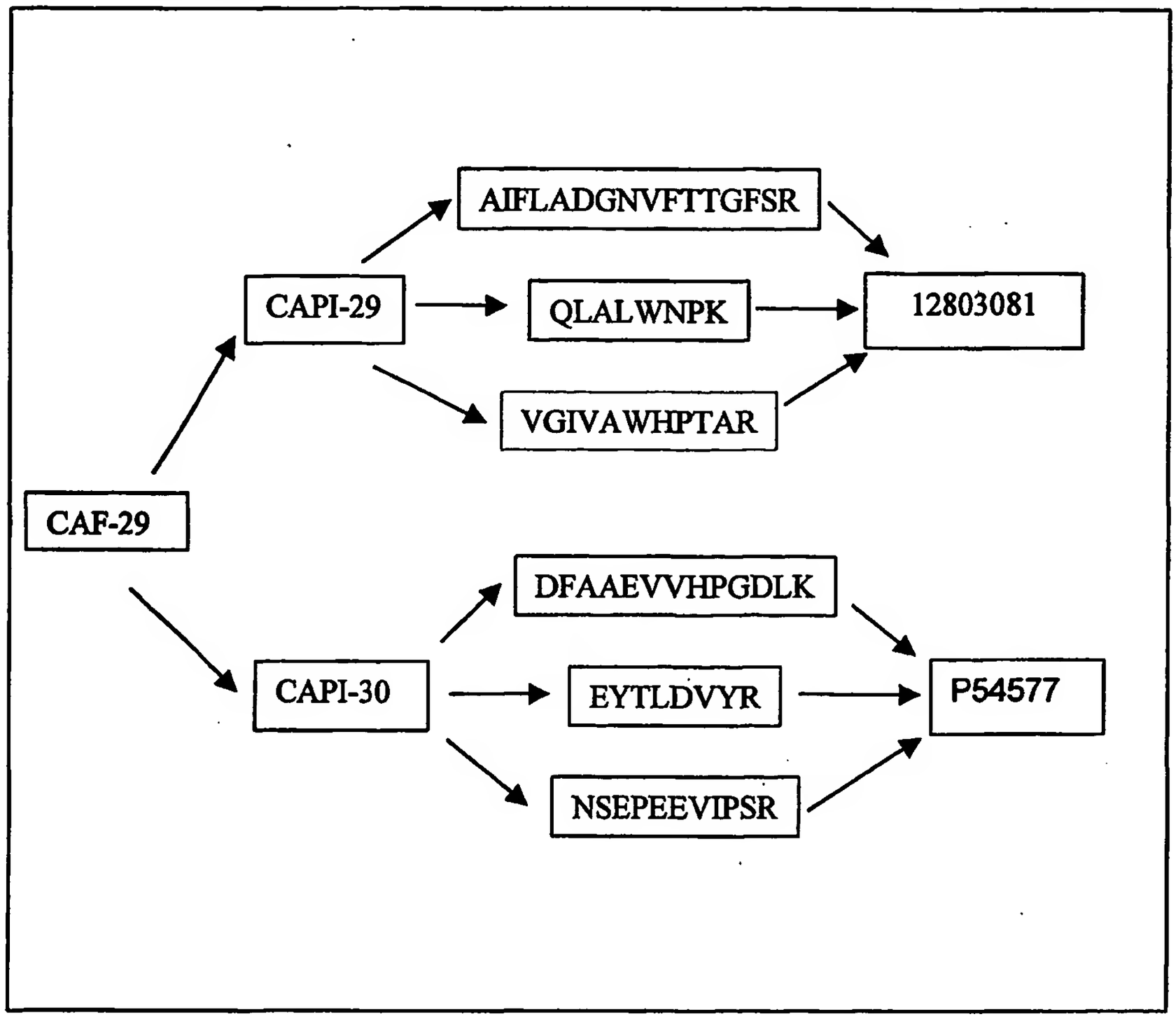
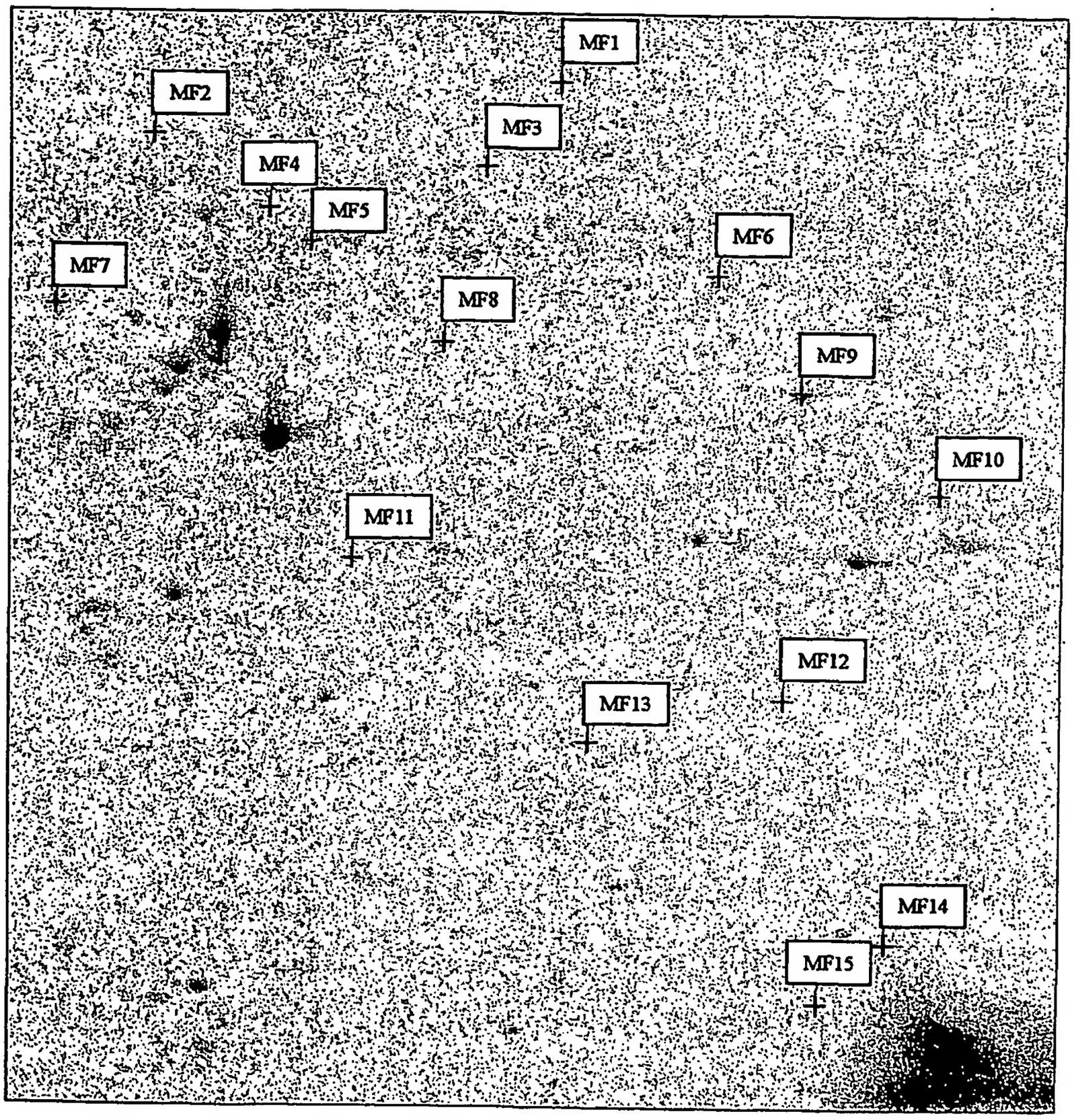


**Figure 2**


MF1
MF2
MF3
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